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(54) Title: PROTEINS, GENES AND THEIR USE FOR DIAGNOSIS AND TREATMENT OF VASCULAR DEMENTIA

(57) Abstract: The present invention provides methods and compositions for screening, diagnosis and prognosis of Vascular Dementia, for monitoring the effectiveness of Vascular Dementia treatment, identifying patients most likely to respond to a particular therapeutic treatment and for drug development. Vascular Dementia-Associated Features (VFs), detectable by two-dimensional electrophoresis of cerebrospinal fluid, serum or plasma are described. The invention further provides Vascular Dementia-Associated Protein Isoforms (VPIs) detectable in cerebrospinal fluid, serum or plasma, preparations comprising isolated VPIs, antibodies immunospecific for VPIs, and kits comprising the aforesaid.

PROTEINS, GENES AND THEIR USE FOR DIAGNOSIS AND TREATMENT OF VASCULAR DEMENTIA

1. INTRODUCTION

The present invention relates to the identification of proteins and protein isoforms that are associated with Vascular Dementia and its onset and development, and of genes encoding the same, and to their use for *e.g.*, clinical screening, diagnosis, prognosis, therapy and prophylaxis, as well as for drug screening and drug development.

2. BACKGROUND OF THE INVENTION

Vascular dementia is the second most common cause of dementia in the US and Europe and a very heterogeneous disease with many factors contributing to the overall pathogenesis. Eight major types of vascular dementia have been identified: 1. Multi-infarct dementia secondary to large cerebral emboli, 2. Strategically placed infarctions causing dementia, 3. Multiple subcortical lacunar lesions secondary to atherosclerosis or degenerative arteriolar changes, 4. Binswanger's disease (arteriosclerotic subcortical leukoencephalopathy), 5. Mixtures of types 1, 2 and 3, 6. Haemorrhagic lesions causing dementia, 7. Subcortical dementia secondary to hereditary factors, and 8. Mixtures of dementia of the Alzheimer's type and vascular dementia (Konno et al. *Drugs Aging* (1997) 11:361-73). A great need exists for an improved diagnosis of vascular dementia. Today, the clinician depends on clinical examinations, the patient's history, and, possibly, brain imaging to recognize signs of vascular dementia such as cerebrovascular damage. Currently diagnosis combines several methods including brain imaging of the injured site via computed tomography (CT) or magnetic resonance imaging (MRI) (Kistler et al. *Stroke* (1984) 15:417-26), duplex and transcranial Doppler methods (Comerota et al. *Surgery* (1981) 6:718-29),and positron emission tomography (Frackowiak and Kjaellman *Neurol Clin North Am* (1983) 1:183-200).

Numerous clinical criteria are used in the diagnosis of vascular dementia VD, resulting in variations the frequency of VD diagnosis depending on the applied criteria (4 clinical definitions of VD are currently used: the Hachinski Ischemic Score (HIS), the Alzheimer Disease Diagnostic and Treatment Centers (ADDTC), National Institute of Neurological Disorders and Stroke-Association Internationale pour la Recherche et l' Enseignement en Neurosciences (NINDS-AIREN), and Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)) (Chui et al. *Arch Neurol* (2000) 57:191-6). Even though standardized neuropsychological assessments facilitate the differential diagnosis of

vascular dementia from Lewy bodies dementia and Alzheimer's disease (Ballard et al. *Dement Geriatr Cogn Disord* (1999) 10:104-8), diagnostic criteria for vascular dementia still require validation (Nyenhuis and Gorelick *J Am Geriatr Soc* (1998) 46:1437-48).

In the majority of neurological disorders like vascular dementias, little is known about a link between changes at a cellular and/or molecular level and nervous system structure and function. In an acute stroke a quick assessment of the cause, severity and chance of progression or recurrence is necessary to ensure an optimal treatment to stabilize or reverse the effects. Few biochemical changes have been identified in vascular dementia. Consequently the identification and characterization of cellular and/or molecular causative defects and neuropathologies are necessary for improved treatment of vascular dementia associated disorders. Due to the possibility of worsening or recurrence, speedy diagnosis would be of great benefit, in particular to categorize the patient as follows:

1. Stroke versus nonstroke such as cerebral tumors and subdural hematoma
2. Hemorrhage versus infraction
3. Specific pathophysiological subtypes of cerebral infarction (Donnan *Lancet* (1992) 339:473).

In particular cerebrovascular dementia often coexists with other causes of dementia (Erkinjuntti *Int Psychogeriatr* (1997) 9 Suppl 1:51-8; discussion 77-83) complicating a proper diagnosis and effective treatment strategies. The majority of vascular dementias are caused by both genetic and environmental factors (Plassman and Breitner *J Am Geriatr Soc* (1996) 44:1242-50), although an increased prevalence of vascular dementia has been demonstrated in the cerebral arteriopathy syndrome, a genetic form of vascular dementia (Salloway and Hong *J Geriatr Psychiatry Neurol* (1998) 11:71-7), apolipoprotein E gene polymorphism in Binswanger's disease and vascular dementia (Higuchi et al. *Clin Genet* (1996) 50:459-61) and hereditary cystatin C amyloid angiopathy (HCCAA) in Icelandic patients and hereditary cerebral haemorrhage with amyloidosis, Dutch type (HCHWA-D) (Wang et al. APMIS (1997) 105:41-7).

Although genetics and genotyping may help to define the heritable risk for Vascular dementia, the utility for diagnosis, prognosis and treatment of vascular dementia may be considerably less. Furthermore, no CNS tissue necessary for any gene expression analysis can be obtained for a living patient under normal circumstances. Proteomic approaches appear most suitable for a molecular dissection of such disease phenotypes in the central nervous system (CNS). The entire CNS is largely inaccessible to meaningful mRNA expression-based analyses of primary human material, since post mortem delays in primary human brain tissue

affects mRNAs more readily than proteins (Edgar et al. *Molecular Psychiatry* (1999) 4:173-17). Given that the CSF bathes the brain, changes in its protein composition may reveal alterations in CNS protein expression pattern causatively or diagnostically linked to the disease. Reasonable amounts of DAPs are secreted or released into body fluids by diseased tissue in the living patient at the onset and/or during progression of the disease. In many cases these alterations will be independent of the genetic makeup of the individual and rather directly related to a set of molecular and cellular alterations contribution to the pathogenic phenotype (Carpenter *J Psychiatr Res* (1998) 32:191-5).

Current treatments of vascular dementia include antithrombic therapies (Crowth and Ginsberg in Stroke, Pathophysiology, Diagnosis, and Management Eds. Barnett, Mohr et al. Year, Churchill Livingston, a division of Harcourt Brace & Company), thrombolytic and defibrinogenating agents (Brott and Hacke in Stroke, supra), antiplatelet agents (Weksler in Stroke, supra) and neuroprotective agents (Gluckmann and Gunn in Neuroprotection in CNS diseases, Eds. Baer and Beal Year, Marcel Dekker, Inc. New York).

Currently vascular dementia has no objective biochemical markers useful for diagnosis and prognosis in living patients. The identification of disease associated proteins (DAPs) in the CSF of vascular dementia patients may provide important insights into disease pathology and opportunities for better diagnosis and treatment strategies. However, currently the majority of DAPs identified in vascular dementia also occur in other diseases, such as the CSF tau protein, CSF neuron-specific enolase and CSF neurofilament light protein (Wallin et al. *Alzheimer Dis Assoc Disord* (1999) 13 Suppl 3, S102-5). Several autopsy series of patients with dementia suggest that a high percentage of patients clinically diagnosed with vascular dementia at autopsy will also be found to have pathological changes consistent with coexisting Alzheimer's disease (Nolan et al. *J Am Geriatr Soc* (1998) 46:597-604; Snowdon et al. JAMA (1997) 277:813-817; Hulette et al. *Neurol* (1997) 48:668-672). Therefore in many cases, differential diagnosis of VD to other dementias, such as Alzheimer's disease, Lewy body dementia remains difficult. Therefore, the specificity and the sensitivity of distinguishing individual neurological disorders as well as acute and chronic CNS disease may require the selection of a repertoire of DAPs rather than an individual protein.

Due to the high rates at which other disorders co-occur with Vascular dementia, the time consuming nature of existing, largely inadequate, tests and their expense it would be highly desirable to measure a substance or substances in samples of cerebrospinal fluid (CSF), blood or urine that would lead to a positive diagnosis of vascular dementia or that would help to exclude vascular dementia from the differential diagnosis.

Therefore, a need exists to identify sensitive and specific biomarkers for the diagnosis, to assess severity and predict the outcome of vascular dementia in living subjects. Additionally, there is a clear need for new therapeutic agents for vascular dementia that work quickly, potently, specifically, and with fewer side effects.

3. SUMMARY OF THE INVENTION

The present invention provides methods and compositions for clinical screening, diagnosis, prognosis, therapy and prophylaxis of Vascular Dementia, for monitoring the effectiveness of Vascular Dementia treatment, for selecting participants in clinical trials, for identifying patients most likely to respond to a particular therapeutic treatment and for screening and development of drugs for treatment of Vascular Dementia.

A first aspect of the invention provides methods for diagnosis of Vascular Dementia that comprise analyzing a sample of cerebrospinal fluid (CSF) by two-dimensional electrophoresis to detect the presence or level of at least one Vascular Dementia-Associated Feature (VF), *e.g.*, one or more of the VFs disclosed herein or any combination thereof. These methods are also suitable for clinical screening, prognosis, monitoring the results of therapy, identifying patients most likely to respond to a particular therapeutic treatment, for drug screening and development, and identification of new targets for drug treatment.

A second aspect of the invention provides methods for diagnosis of Vascular Dementia that comprise detecting in a sample of CSF the presence or level of at least one Vascular Dementia-Associated Protein Isoform (VPI), *e.g.*, one or more of the VPIs disclosed herein or any combination thereof. These methods are also suitable for clinical screening, prognosis, monitoring the results of therapy, identifying patients most likely to respond to a particular therapeutic treatment, drug screening and development, and identification of new targets for drug treatment.

A third aspect of the invention provides antibodies, *e.g.* monoclonal and polyclonal antibodies capable of immunospecific binding to a VPI, *e.g.*, a VPI disclosed herein.

A fourth aspect of the invention provides a preparation comprising an isolated VPI, *i.e.*, a VPI free from proteins or protein isoforms having a significantly different isoelectric point or a significantly different apparent molecular weight from the VPI.

A fifth aspect of the invention provides methods of treating Vascular Dementia, comprising administering to a subject a therapeutically effective amount of an agent that modulates (*e.g.*, upregulates or downregulates) the expression or activity (*e.g.* enzymatic or binding activity), or both, of a VPI in subjects having Vascular Dementia, in order to prevent

or delay the onset or development of Vascular Dementia, to prevent or delay the progression of Vascular Dementia, or to ameliorate the symptoms of Vascular Dementia.

A sixth aspect of the invention provides methods of screening for agents that modulate (*e.g.*, upregulate or downregulate) a characteristic of, *e.g.*, the expression or the enzymatic or binding activity, of a VPI, a VPI analog, or a VPI-related polypeptide.

3.1. Definitions

The term "VPI analog" as used herein refers to a polypeptide that possesses a similar or identical function as a VPI but need not necessarily comprise an amino acid sequence that is similar or identical to the amino acid sequence of the VPI, or possess a structure that is similar or identical to that of the VPI. As used herein, an amino acid sequence of a polypeptide is "similar" to that of a VPI if it satisfies at least one of the following criteria: (a) the polypeptide has an amino acid sequence that is at least 30% (more preferably, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99%) identical to the amino acid sequence of the VPI; (b) the polypeptide is encoded by a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence encoding at least 5 amino acid residues (more preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, or at least 150 amino acid residues) of the VPI; or (c) the polypeptide is encoded by a nucleotide sequence that is at least 30% (more preferably, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99%) identical to the nucleotide sequence encoding the VPI. As used herein, a polypeptide with "similar structure" to that of a VPI refers to a polypeptide that has a similar secondary, tertiary or quarternary structure as that of the VPI. The structure of a polypeptide can determined by methods known to those skilled in the art, including but not limited to, X-ray crystallography, nuclear magnetic resonance, and crystallographic electron microscopy.

The term "VPI fusion protein" as used herein refers to a polypeptide that comprises (i) an amino acid sequence of a VPI, a VPI fragment, a VPI-related polypeptide or a fragment of a VPI-related polypeptide and (ii) an amino acid sequence of a heterologous polypeptide (*i.e.*, a non-VPI, non-VPI fragment or non-VPI- related polypeptide).

The term "VPI homolog" as used herein refers to a polypeptide that comprises an amino acid sequence similar to that of a VPI but does not necessarily possess a similar or identical function as the VPI.

The term "VPI ortholog" as used herein refers to a non-human polypeptide that (i) comprises an amino acid sequence similar to that of a VPI and (ii) possesses a similar or identical function to that of the VPI.

The term "VPI-related polypeptide" as used herein refers to a VPI homolog, a VPI analog, an isoform of VPI, a VPI ortholog, or any combination thereof.

The term "derivative" as used herein refers to a polypeptide that comprises an amino acid sequence of a second polypeptide which has been altered by the introduction of amino acid residue substitutions, deletions or additions. The derivative polypeptide possess a similar or identical function as the second polypeptide.

The term "fragment" as used herein refers to a peptide or polypeptide comprising an amino acid sequence of at least 5 amino acid residues (preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, at least 150 amino acid residues, at least 175 amino acid residues, at least 200 amino acid residues, or at least 250 amino acid residues) of the amino acid sequence of a second polypeptide. The fragment of a VPI may or may not possess a functional activity of the second polypeptide.

The term "fold change" includes "fold increase" and "fold decrease" and refers to the relative increase or decrease in abundance of a VF or the relative increase or decrease in expression or activity of a polypeptide (*e.g.* a VPI) in a first sample or sample set compared to a second sample (or sample set). A VF or polypeptide fold change may be measured by any technique known to those of skill in the art, however the observed increase or decrease will vary depending upon the technique used. Preferably, fold change is determined herein as described in the Examples *infra*.

The term "isoform" as used herein refers to variants of a polypeptide that are encoded by the same gene, but that differ in their pI or MW, or both. Such isoforms can differ in their amino acid composition (*e.g.* as a result of alternative splicing or limited proteolysis) and in addition, or in the alternative, may arise from differential post-translational modification (*e.g.*, glycosylation, acylation, phosphorylation). As used herein, the term "isoform" also refers to a protein that exists in only a single form, *i.e.*, it is not expressed as several variants.

The term "modulate" when used herein in reference to expression or activity of a VPI or a VPI-related polypeptide refers to the upregulation or downregulation of the expression or activity of the VPI or a VPI-related polypeptide. Based on the present disclosure, such modulation can be determined by assays known to those of skill in the art or described herein.

The percent identity of two amino acid sequences or of two nucleic acid sequences is determined by aligning the sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in the first sequence for best alignment with the sequence) and comparing the amino acid residues or nucleotides at corresponding positions. The "best alignment" is an alignment of two sequences which results in the highest percent identity. The percent identity is determined by the number of identical amino acid residues or nucleotides in the sequences being compared (*i.e.*, % identity = # of identical positions/total # of positions x 100).

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul *Proc. Natl. Acad. Sci. USA* (1990) 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. The NBLAST and XBLAST programs of Altschul et al, *J. Mol. Biol.* (1990) 215:403-410 have incorporated such an algorithm. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al, *Nucleic Acids Res.* (1997) 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Another example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). The ALIGN program (version 2.0) which is part of the GCG sequence alignment software package has incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torellis and Robotti *Comput. Appl. Biosci.* (1994) 10:3-5; and FASTA described in Pearson and Lipman *Proc. Natl. Acad. Sci. USA* (1988)

85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is an image obtained from 2-dimensional electrophoresis of human CSF, which has been annotated to identify twelve landmark features, designated CSF1 to CSF12.

5. DETAILED DESCRIPTION OF THE INVENTION

The invention described in detail below provides methods and compositions for clinical screening, diagnosis and prognosis of Vascular Dementia in a mammalian subject for identifying patients most likely to respond to a particular therapeutic treatment, for monitoring the results of Vascular Dementia therapy, for drug screening and drug development. The invention also encompasses the administration of therapeutic compositions to a mammalian subject to treat or prevent Vascular Dementia. The mammalian subject may be a non-human mammal, but is preferably human, more preferably a human adult, *i.e.* a human subject at least 21 (more preferably at least 35, at least 50, at least 60, at least 70, or at least 80) years old. For clarity of disclosure, and not by way of limitation, the invention will be described with respect to the analysis of CSF samples. However, as one skilled in the art will appreciate, the assays and techniques described below can be applied to other types of samples, including a body fluid (*e.g.* blood, serum, plasma, saliva or urine), a tissue sample from a subject at risk of having or developing Vascular Dementia (*e.g.* a biopsy such as a brain biopsy) or homogenate thereof. The methods and compositions of the present invention are useful for screening, diagnosis and prognosis of a living subject, but may also be used for postmortem diagnosis in a subject, for example, to identify family members of the subject who are at risk of developing the same disease.

As used herein, cerebrospinal fluid (CSF) refers to the fluid that surrounds the bulk of the central nervous system, as described in Physiological Basis of Medical Practice (J.B. West, ed., Williams and Wilkins, Baltimore, MD 1985). CSF includes ventricular CSF and lumbar CSF. As used herein, the term "serum" refers to the supernatant fluid produced by clotting and centrifugal sedimentation of a blood sample. As used herein, the term "plasma" refers to the supernatant fluid produced by inhibition of clotting (for example, by citrate or EDTA) and centrifugal sedimentation of a blood sample. The term "blood" as used herein includes serum and plasma.

5.1 Vascular Dementia-Associated Features (VFs)

In one aspect of the invention, two-dimensional electrophoresis is used to analyze CSF from a subject, preferably a living subject, in order to detect or quantify the expression of one or more Vascular Dementia-Associated Features (VFs) for screening, prevention or diagnosis of Vascular Dementia, to determine the prognosis of a subject having Vascular Dementia, to monitor progression of Vascular Dementia, to monitor the effectiveness of Vascular Dementia therapy, for identifying patients most likely to respond to a particular therapeutic treatment, or for drug development. As used herein, "two-dimensional electrophoresis" (2D-electrophoresis) means a technique comprising isoelectric focusing, followed by denaturing electrophoresis; this generates a two- dimensional gel (2D-gel) containing a plurality of separated proteins. Preferably, the step of denaturing electrophoresis uses polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Especially preferred are the highly accurate and automatable methods and apparatus ("the Preferred Technology") described in International Application No. 97GB3307 (published as WO 98/23950) and in U.S. Application No. 08/980,574, both filed December 1, 1997, each of which is incorporated herein by reference in its entirety with particular reference to the protocol at pages 23-35. Briefly, the Preferred Technology provides efficient, computer-assisted methods and apparatus for identifying, selecting and characterizing biomolecules (*e.g.* proteins, including glycoproteins) in a biological sample. A two-dimensional array is generated by separating biomolecules on a two-dimensional gel according to their electrophoretic mobility and isoelectric point. A computer-generated digital profile of the array is generated, representing the identity, apparent molecular weight, isoelectric point, and relative abundance of a plurality of biomolecules detected in the two-dimensional array, thereby permitting computer-mediated comparison of profiles from multiple biological samples, as well as computer aided excision of separated proteins of interest.

A preferred scanner for detecting fluorescently labelled proteins is described in WO 96/36882 and in the Ph.D. thesis of David A. Basiji, entitled "Development of a High-throughput Fluorescence Scanner Employing Internal Reflection Optics and Phase-sensitive Detection (Total Internal Reflection, Electrophoresis)", University of Washington (1997), Volume 58/12-B of Dissertation Abstracts International, page 6686, the contents of each of which are incorporated herein by reference. These documents describe an image scanner designed specifically for automated, integrated operation at high speeds. The scanner can image gels that have been stained with fluorescent dyes or silver stains, as well as storage phosphor screens. The Basiji thesis provides a phase-sensitive detection system for

discriminating modulated fluorescence from baseline noise due to laser scatter or homogeneous fluorescence, but the scanner can also be operated in a non-phase-sensitive mode. This phase-sensitive detection capability would increase the sensitivity of the instrument by an order of magnitude or more compared to conventional fluorescence imaging systems. The increased sensitivity would reduce the sample-preparation load on the upstream instruments while the enhanced image quality simplifies image analysis downstream in the process.

A more highly preferred scanner is the Apollo 2 scanner (Oxford Glycosciences, Oxford, UK), which is a modified version of the above described scanner. In the Apollo 2 scanner, the gel is transported through the scanner on a precision lead-screw drive system. This is preferable to laying the glass plate on the belt-driven system that is described in the Basiji thesis, as it provides a reproducible means of accurately transporting the gel past the imaging optics.

In the Apollo 2 scanner, the gel is secured against three alignment stops that rigidly hold the glass plate in a known position. By doing this in conjunction with the above precision transport system, the absolute position of the gel can be predicted and recorded. This ensures that co-ordinates of each feature on the gel can be determined more accurately and communicated, if desired, to a cutting robot for excision of the feature. In the Apollo 2 scanner, the carrier that holds the gel has four integral fluorescent markers for use to correct the image geometry. These markers are a quality control feature that confirms that the scanning has been performed correctly.

In comparison to the scanner described in the Basiji thesis, the optical components of the Apollo 2 scanner have been inverted. In the Apollo 2 scanner, the laser, mirror, waveguide and other optical components are above the glass plate being scanned. The scanner described in the Basiji thesis has these components underneath. In the Apollo 2 scanner, the glass plate is mounted onto the scanner gel side down, so that the optical path remains through the glass plate. By doing this, any particles of gel that may break away from the glass plate will fall onto the base of the instrument rather than into the optics. This does not affect the functionality of the system, but increases its reliability.

Still more preferred is the Apollo 3 scanner, in which the signal output is digitized to the full 16-bit data without any peak saturation or without square root encoding of the signal. A compensation algorithm has also been applied to correct for any variation in detection sensitivity along the path of the scanning beam. This variation is due to anomalies in the optics and differences in collection efficiency across the waveguide. A calibration is

performed using a perspex plate with an even fluorescence throughout. The data received from a scan of this plate are used to determine the multiplication factors needed to increase the signal from each pixel level to a target level. These factors are then used in subsequent scans of gels to remove any internal optical variations.

As used herein, the term "feature" refers to a spot detected in a 2D gel, and the term "Vascular Dementia-Associated Feature" (VF) refers to a feature that is differentially present in a sample (*e.g.* a sample of CSF) from a subject having Vascular Dementia compared with a sample (*e.g.* a sample of CSF) from a subject free from Vascular Dementia. As used herein, a feature (or a protein isoform of VPI, as defined *infra*) is "differentially present" in a first sample with respect to a second sample when a method for detecting the feature, isoform or VPI (*e.g.*, 2D electrophoresis or an immunoassay) gives a different signal when applied to the first and second samples. A feature, isoform or VPI is "increased" in the first sample with respect to the second if the method of detection indicates that the feature, isoform or VPI is more abundant in the first sample than in the second sample, or if the feature, isoform or VPI is detectable in the first sample and undetectable in the second sample. Conversely, a feature, isoform or VPI is "decreased" in the first sample with respect to the second if the method of detection indicates that the feature, isoform or VPI is less abundant in the first sample than in the second sample or if the feature, isoform or VPI is undetectable in the first sample and detectable in the second sample.

Preferably, the relative abundance of a feature in two samples is determined in two steps. First, the signal obtained upon detecting the feature in a sample is normalized by reference to a suitable background parameter, *e.g.*, (a) to the total protein in the sample being analyzed (*e.g.*, total protein loaded onto a gel); (b) to an Expression Reference Feature (ERF) *i.e.*, a feature whose abundance is invariant, within the limits of variability of the Preferred Technology, in the population of subjects being examined, *e.g.* the ERFs disclosed below, or (c) more preferably to the total signal detected from all proteins in the sample.

Secondly, the normalized signal for the feature in one sample or sample set is compared with the normalized signal for the same feature in another sample or sample set in order to identify features that are "differentially present" in the first sample (or sample set) with respect to the second.

The VFs disclosed herein have been identified by comparing CSF samples from subjects having Vascular Dementia against CSF samples from subjects free from Vascular Dementia. Subjects free from Vascular Dementia include subjects with no known disease or

condition (normal subjects) and subjects with diseases (including neurological and neurodegenerative diseases) other than Vascular Dementia.

Two groups of VFs have been identified through the methods and apparatus of the Preferred Technology. The first group consists of VFs that are decreased in the CSF of subjects having Vascular Dementia as compared with the CSF of subjects free from Vascular Dementia. These VFs can be described by apparent molecular weight (MW) and isoelectric point (pI) as provided in Table I.

Table I. VFs Decreased in CSF of Subjects Having Vascular Dementia

VF#	pI	MW (Da)	Fold Decrease	Rank Sum P-Value
VF-4	8.89	11749	>100	
VF-5	9.16	20681	>100	
VF-12	7.62	70511	58.28	
VF-13	6.18	105482	34.78	
VF-14	4.65	102603	16.85	
VF-15	5.73	14776	30.68	
VF-16	4.65	32509	8.11	
VF-17	9.50	13985	10.15	
VF-18	6.45	94205	6.52	
VF-19	8.54	54625	5.28	
VF-20	6.30	73287	5.58	
VF-21	6.35	164223	4.70	
VF-22	9.04	11790	16.20	0.03689
VF-23	6.58	93680	4.60	
VF-24	7.51	37524	4.83	
VF-25	9.32	13044	8.57	
VF-26	9.18	48532	4.24	
VF-27	9.76	13546	7.04	
VF-29	5.26	11388	7.37	
VF-30	4.65	10120	4.15	
VF-31	7.80	29113	3.80	
VF-32	6.92	109447	7.33	
VF-33	4.34	10961	8.73	
VF-34	4.12	63272	7.14	
VF-35	5.65	13084	6.84	
VF-36	8.99	61111	5.20	

VF#	pI	MW (Da)	Fold Decrease	Rank Sum P-Value
VF-37	9.80	23795	4.30	
VF-38	8.79	22458	6.43	
VF-41	4.91	38741	3.28	0.01219
VF-42	6.15	25232	2.74	0.03671
VF-43	5.67	48092	2.74	0.01219
VF-44	5.65	150534	2.09	0.03689
VF-45	4.53	11037	2.11	0.03734
VF-46	4.86	38741	2.61	0.01219
VF-47	5.49	57515	2.65	0.01996
VF-48	4.99	15072	2.24	0.02157
VF-50	5.48	55124	2.43	0.01219
VF-51	6.53	75865	2.01	0.03734
VF-52	5.37	123390	2.14	0.03671
VF-53	6.61	72071	1.82	0.03671
VF-54	4.57	13499	1.98	0.03671
VF-55	5.30	49423	1.94	0.01219
VF-57	9.18	39998	1.61	0.03671
VF-58	4.86	135312	1.95	0.03671
VF-60	5.66	21021	1.49	0.02157
VF-64	5.77	52354	1.45	0.01219
VF-66	5.01	25963	1.47	0.03671
VF-68	5.66	63184	1.46	0.01219
VF-171	8.40	14528	53.08	
VF-172	9.05	18350	41.40	
VF-173	4.19	34949	36.28	
VF-174	5.29	86159	28.38	
VF-175	4.83	184426	21.03	
VF-176	4.17	34311	18.79	
VF-177	4.51	50790	14.64	
VF-178	4.66	12080	14.51	
VF-179	9.19	37198	12.72	
VF-180	9.12	38200	12.34	
VF-181	9.65	65654	11.58	
VF-182	4.76	185225	11.13	
VF-183	4.58	52595	10.72	
VF-184	5.32	65751	10.07	

VF#	pI	MW (Da)	Fold Decrease	Rank Sum P-Value
VF-185	4.39	89591	8.81	
VF-186	9.16	33591	8.80	
VF-187	6.87	66392	8.80	
VF-188	7.89	29058	7.13	
VF-189	8.54	34332	6.99	
VF-190	7.92	77836	6.73	
VF-191	6.27	186027	5.77	
VF-192	9.62	88830	5.64	
VF-193	9.82	67749	5.24	
VF-194	5.08	26202	5.21	
VF-195	7.14	31448	5.14	
VF-196	9.68	35740	5.11	
VF-197	5.00	33473	5.03	
VF-198	6.66	45483	4.95	
VF-199	5.74	32454	4.87	
VF-200	4.70	19478	4.70	
VF-201	9.00	32275	4.66	
VF-202	6.38	30254	4.65	
VF-203	4.12	93680	4.62	
VF-204	7.13	32002	4.42	
VF-205	7.49	51535	4.41	
VF-206	4.30	81939	4.36	
VF-207	8.01	34096	4.36	
VF-208	5.57	58693	4.34	
VF-209	9.86	34695	4.33	
VF-210	4.67	65033	4.21	
VF-211	9.36	16491	4.16	
VF-212	9.39	11427	4.11	
VF-213	5.29	54625	4.04	
VF-214	4.94	43667	4.04	
VF-215	9.21	21632	3.95	
VF-216	5.05	153158	3.77	
VF-217	5.85	19316	3.76	
VF-218	5.61	74489	3.74	
VF-219	8.97	17091	3.68	
VF-220	4.16	36450	3.66	

VF#	pI	MW (Da)	Fold Decrease	Rank Sum P-Value
VF-221	7.27	41723	3.07	
VF-222	6.24	102603	2.82	
VF-223	7.89	57515	2.55	
VF-224	4.67	94138	2.54	
VF-225	7.26	16614	2.48	
VF-226	5.19	12080	2.36	
VF-227	5.04	40716	2.33	0.03038
VF-228	5.08	91613	2.32	0.03734
VF-229	6.21	12903	2.32	
VF-230	5.82	50026	2.27	0.03671
VF-231	4.56	20268	2.24	
VF-232	8.16	34096	2.18	
VF-233	5.03	181267	2.15	
VF-234	5.58	63762	2.10	0.03671
VF-235	4.94	134070	2.04	0.03671
VF-236	6.89	42326	1.99	0.03689
VF-237	4.93	102603	1.97	
VF-238	8.90	15083	1.90	
VF-239	4.26	75782	1.80	
VF-240	6.57	28136	1.77	0.03734
VF-241	4.95	45574	1.76	0.03689
VF-242	4.82	81483	1.72	0.02157
VF-243	4.31	88597	1.71	
VF-244	5.38	26392	1.68	
VF-245	6.18	187641	1.65	
VF-246	5.24	88817	1.65	
VF-247	4.82	30552	1.63	
VF-248	5.48	51880	1.61	0.03689
VF-249	5.10	27209	1.61	
VF-250	6.00	49723	1.59	0.03671
VF-251	6.64	123390	1.58	0.03671
VF-252	5.62	50026	1.58	
VF-253	6.57	20336	1.57	
VF-254	4.46	32643	1.53	0.03671
VF-255	5.05	160613	1.48	
VF-256	6.77	34594	1.44	

VF#	pI	MW (Da)	Fold Decrease	Rank Sum P-Value
VF-257	7.51	27636	1.44	
VF-258	5.19	91103	1.42	0.02157
VF-259	6.28	24374	1.29	
VF-260	7.47	19095	1.21	
VF-261	5.82	41902	1.17	
VF-262	8.17	12814	1.16	
VF-263	5.16	26050	1.15	
VF-264	4.74	30882	1.11	
VF-265	7.96	42551	1.07	

Where p values are given in Table I, the statistical technique used was the Wilcoxon Rank-Sum test as described in method (a) of Section 6.1.13, Statistical Analysis of the Profiles. Where no p value is reported, the method used to select these features was on the basis of a significant fold change or qualitative presence or absence alone as described in methods (b) and (c) of Section 6.1.13 Statistical Analysis of the Profiles.

The second group consists of VFs that are increased in the CSF of subjects having Vascular Dementia as compared with the CSF of subjects free from Vascular Dementia. These VFs can be described by MW and pI as follows:

Table II. VFs Increased in CSF of Subjects Having Vascular Dementia

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-92	9.80	18109	62.10	
VF-93	9.59	18169	55.37	
VF-94	9.74	56994	27.53	
VF-95	9.47	37090	15.80	
VF-96	4.34	150277	11.95	
VF-97	7.24	11749	40.11	
VF-98	7.48	11668	29.20	
VF-99	4.29	40288	19.05	
VF-100	7.50	55738	18.91	
VF-101	4.32	38629	11.85	
VF-102	5.91	64736	28.05	
VF-103	5.26	10405	17.77	
VF-104	5.82	11533	15.41	

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-105	4.45	30662	16.52	
VF-106	3.96	60192	14.22	
VF-107	6.31	17820	13.10	
VF-108	7.88	11369	14.95	
VF-109	6.18	15749	10.74	
VF-110	7.27	14163	16.78	
VF-111	10.21	31005	6.65	
VF-112	6.81	57414	6.16	
VF-113	5.84	39556	7.96	
VF-114	9.81	41481	12.47	
VF-115	5.79	16609	6.00	
VF-116	6.08	10122	8.60	
VF-118	5.79	14996	11.57	
VF-120	6.17	63376	8.02	
VF-121	7.05	11388	7.81	
VF-122	7.44	26066	9.79	
VF-123	6.65	13831	9.31	
VF-124	5.87	45258	6.48	
VF-125	6.80	65526	7.82	
VF-126	8.02	11130	7.59	
VF-127	9.80	18843	9.10	
VF-128	5.61	135816	9.48	
VF-129	5.59	158545	8.51	
VF-130	4.84	118262	6.15	
VF-131	4.39	89827	5.88	
VF-132	6.54	13783	6.07	
VF-134	4.77	99610	8.21	
VF-135	6.45	20882	5.35	0.03689
VF-136	9.58	20268	5.98	0.01996
VF-137	7.26	12594	5.11	0.03689
VF-138	9.22	16179	5.22	0.01219
VF-139	9.22	19032	5.08	0.01945
VF-140	6.11	31600	3.59	0.03689
VF-141	4.40	146682	4.64	0.01996
VF-142	6.35	19414	4.29	0.01219
VF-143	9.58	21021	4.35	0.03689

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-144	7.42	56136	3.85	0.02157
VF-145	8.16	59646	4.57	0.03689
VF-146	6.20	87509	2.77	0.03689
VF-147	6.23	12206	3.82	0.01996
VF-148	5.66	146050	3.92	0.03038
VF-149	6.56	20744	3.18	0.03734
VF-150	7.48	59646	3.79	0.03671
VF-151	7.47	22090	3.88	0.02157
VF-152	6.86	50636	3.08	0.03734
VF-153	6.74	54791	3.16	0.01219
VF-154	7.27	94587	3.16	0.01996
VF-155	6.13	88018	2.71	0.01219
VF-156	5.97	14520	3.04	0.02157
VF-157	9.24	21021	2.92	0.01996
VF-158	9.26	21908	2.88	0.01996
VF-159	9.78	29583	2.61	0.01996
VF-160	6.21	67544	2.11	0.01219
VF-161	6.11	74524	2.11	0.02157
VF-162	6.08	27741	2.10	0.01996
VF-163	6.53	10226	2.69	0.01996
VF-164	8.44	19222	2.41	0.01996
VF-166	5.02	67749	1.71	0.03734
VF-168	5.22	13359	1.55	0.02157
VF-170	4.62	28747	1.35	0.03689
VF-266	7.43	29689	26.49	
VF-267	5.51	76637	21.60	
VF-268	8.10	55548	20.80	
VF-269	7.69	31809	18.25	
VF-270	6.03	89654	17.00	
VF-271	9.08	28954	12.44	
VF-272	6.04	40937	12.28	
VF-273	5.69	16547	10.01	
VF-274	4.12	13577	9.04	
VF-275	4.23	39766	9.01	
VF-276	7.19	20961	8.61	
VF-277	4.24	41761	8.59	

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-278	9.81	14171	8.58	
VF-279	4.34	30225	8.28	
VF-280	9.31	11137	8.22	
VF-281	10.06	11587	8.18	
VF-282	5.98	45728	7.95	
VF-283	10.04	14024	7.72	
VF-284	9.98	10735	7.72	
VF-285	5.79	27098	7.67	
VF-286	10.03	17060	7.52	
VF-287	7.83	23298	7.38	
VF-288	9.82	59646	7.09	
VF-289	5.67	12175	6.89	
VF-290	9.81	10923	6.87	
VF-291	9.91	27009	6.54	
VF-292	4.83	56582	6.41	
VF-293	6.05	14637	6.36	
VF-294	6.66	14286	6.35	
VF-295	4.39	30225	6.19	
VF-296	5.14	19356	6.14	
VF-297	5.63	158545	6.06	
VF-298	6.40	13915	6.05	
VF-299	5.49	158545	6.01	
VF-300	6.29	44357	5.98	
VF-301	5.53	10673	5.71	
VF-302	7.65	61670	5.65	
VF-303	9.83	39766	5.36	0.03689
VF-304	5.39	28172	4.94	
VF-305	4.69	53640	4.86	
VF-306	5.85	45882	4.59	0.02157
VF-307	9.19	56533	4.20	
VF-308	6.48	42899	4.01	
VF-309	6.03	13175	3.85	
VF-310	6.61	11467	3.56	
VF-311	6.15	45719	3.35	
VF-312	6.29	93680	3.34	
VF-313	4.53	30225	3.33	

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-314	6.98	59466	3.22	0.03689
VF-315	5.71	45728	3.08	
VF-316	6.08	30920	3.00	0.03734
VF-317	6.09	82463	2.94	
VF-318	6.13	178161	2.79	0.03734
VF-319	7.38	59828	2.74	0.02157
VF-320	5.78	148321	2.72	
VF-321	7.31	11037	2.66	
VF-322	5.20	11309	2.61	
VF-323	5.90	44068	2.51	
VF-324	6.39	44664	2.49	0.04975
VF-325	6.28	178161	2.43	0.01219
VF-326	7.07	39307	2.40	
VF-327	6.05	55588	2.22	
VF-328	6.66	65725	2.15	0.03689
VF-329	9.05	19478	2.07	
VF-330	4.77	18049	2.03	
VF-331	6.28	67135	2.01	0.03671
VF-332	6.22	23973	2.00	
VF-333	7.14	32549	1.93	
VF-334	6.06	11270	1.90	0.03671
VF-335	6.70	38112	1.89	
VF-336	5.75	24567	1.86	
VF-337	6.73	62107	1.83	
VF-338	5.84	11062	1.83	
VF-339	5.75	34874	1.83	
VF-340	6.07	65130	1.76	
VF-341	7.01	40510	1.74	0.03655
VF-342	8.16	24182	1.71	0.03671
VF-343	5.47	51028	1.69	
VF-344	6.66	19935	1.68	0.03689
VF-345	5.50	112518	1.63	
VF-346	5.73	22738	1.62	
VF-347	7.40	114396	1.51	
VF-348	5.10	29902	1.49	
VF-349	6.35	53084	1.48	0.03734

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-350	4.64	119650	1.47	
VF-351	9.07	23405	1.46	
VF-352	6.57	19011	1.42	
VF-353	6.69	39193	1.40	
VF-354	4.36	12420	1.37	
VF-355	6.55	164120	1.34	
VF-356	5.44	53312	1.30	
VF-357	5.62	26300	1.28	
VF-358	4.63	29662	1.28	
VF-359	5.02	80131	1.26	
VF-360	4.57	30225	1.25	
VF-361	4.55	24374	1.24	
VF-362	7.29	20444	1.20	
VF-363	6.17	74110	1.18	
VF-364	4.94	171366	1.12	
VF-365	4.41	24762	1.11	
VF-366	6.38	38294	1.10	
VF-367	5.11	24111	1.08	
VF-368	5.14	13240	1.07	
VF-369	6.84	42786	1.05	
VF-370	4.40	27223	1.04	
VF-371	6.46	34796	1.03	
VF-372	6.76	25490	1.02	

Where p values are given in Table II, the statistical technique used was the Wilcoxon Rank-Sum test as described in method (a) of Section 6.1.13 Statistical Analysis of the Profiles. Where no p value is reported, the method used to select these features was on the basis of fold change or qualitative presence or absence alone as described in methods (b) and (c) of Section 6.1.13 Statistical Analysis of the Profiles.

For any given VF, the signal obtained upon analyzing CSF from subjects having Vascular Dementia relative to the signal obtained upon analyzing CSF from subjects free from Vascular Dementia will depend upon the particular analytical protocol and detection technique that is used. Accordingly, the present invention contemplates that each laboratory will, based

on the present description, establish a reference range for each VF in subjects free from Vascular Dementia according to the analytical protocol and detection technique in use, as is conventional in the diagnostic art. Preferably, at least one control positive CSF sample from a subject known to have Vascular Dementia or at least one control negative CSF sample from a subject known to be free from Vascular Dementia (and more preferably both positive and negative control samples) are included in each batch of test samples analyzed. In one embodiment, the level of expression of a feature is determined relative to a background value, which is defined as the level of signal obtained from a proximal region of the image that (a) is equivalent in area to the particular feature in question; and (b) contains no discernable protein feature. The reference range, depending upon the method of detection used and the conditions under which detection is carried out, can include no feature or isoform present, or non-detectable levels of feature or isoform present. Proteins described by pI and MW provided in Tables I and II can be identified by searching 2D-PAGE databases with those pI and MW values. Examples of such databases are provided on the ExPASy Molecular Biology Server (http://www.expasy.ch) under the "SWISS-2DPAGE" section, and other databases are further referenced on this server. Such databases typically provide interactive 2D gels images for a given set of sample and preparation protocol, and the skilled artisan can obtain information relevant to a given feature by pointing and clicking the appropriate section of the image.

In a preferred embodiment, the signal associated with a VF in the CSF of a subject (*e.g.*, a subject suspected of having or known to have Vascular Dementia) is normalized with reference to one or more ERFs detected in the same 2D gel. As will be apparent to one of ordinary skill in the art, such ERFs may readily be determined by comparing different samples using the Preferred Technology. Suitable ERFs include (but are not limited to) that described in the following table.

Table III. Expression Reference Features

ERF#	pI	MW (Da)
ERF-1	7.34	30993
ERF-2	4.86	60009

As those of skill in the art will readily appreciate, the measured MW and pI of a given feature or protein isoform will vary to some extent depending on the precise protocol used for each step of the 2D electrophoresis and for landmark matching. As used herein, the terms

"MW" and "pI" are defined, respectively, to mean the apparent molecular weight and the apparent isoelectric point of a feature or protein isoform as measured in exact accordance with the Reference Protocol identified in Section 6 below. When the Reference Protocol is followed and when samples are run in duplicate or a higher number of replicates, variation in the measured mean pI of a VF or VPI is typically less than 3% and variation in the measured mean MW of a VF or VPI is typically less than 5%. Where the skilled artisan wishes to deviate from the Reference Protocol, calibration experiments should be performed to compare the MW and pI for each VF or protein isoform as detected (a) by the Reference Protocol and (b) by the deviant protocol.

VFs can be used for detection, prognosis, diagnosis, or monitoring of Vascular Dementia, or for identifying patients most likely to respond to a specific therapeutic treatment, or for drug development. In one embodiment of the invention, CSF from a subject (*e.g.*, a subject suspected of having Vascular Dementia) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following VFs: VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24, VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194, VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265. A decreased abundance of said one or more VFs in the CSF from the subject relative to CSF from a subject or subjects free from Vascular Dementia (*e.g.*, a control sample or a previously determined reference range) indicates the presence of Vascular Dementia.

In another embodiment of the invention, CSF from a subject is analyzed by 2D electrophoresis for quantitative detection of one or more of the following VFs: VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-

115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, VF-372. An increased abundance of said one or more VFs in the CSF from the subject relative to CSF from a subject or subjects free from Vascular Dementia (*e.g.*, a control sample or a previously determined reference range) indicates the presence of Vascular Dementia.

In yet another embodiment, CSF from a subject is analyzed by 2D electrophoresis for quantitative detection of (a) one or more VFs or any combination of them, whose decreased abundance indicates the presence of Vascular Dementia, *i.e.*, VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24, VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194, VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265; and (b) one or more VFs or any

combination of them, whose increased abundance indicates the presence of Vascular Dementia *i.e.*, VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, VF-372.

In yet another embodiment of the invention, CSF from a subject is analyzed by 2D electrophoresis for quantitative detection of one or more of the following VFs: VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24, VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194,

VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, VF-372 wherein the ratio of the one or more VFs relative to an Expression Reference Feature (ERF) indicates whether Vascular Dementia is present. In a specific embodiment, a decrease in one or more VF/ERF ratios in a test sample relative to the VF/ERF ratios in a control sample or a reference range indicates the presence of Vascular Dementia; VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24, VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194, VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265 are suitable VFs

for this purpose. In another specific embodiment, an increase in one or more VF/ERF ratios in a test sample relative to the VF/ERF ratios in a control sample or a reference range indicates the presence of Vascular Dementia; VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, VF-372 are suitable VFs for this purpose.

In a further embodiment of the invention, CSF from a subject is analyzed by 2D electrophoresis for quantitative detection of (a) one or more VFs, or any combination of them, whose decreased VF/ERF ratio(s) in a test sample relative to the VF/ERF ratio(s) in a control sample indicates the presence of Vascular Dementia, *i.e.*, VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24, VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194, VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-

238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265; (b) one or more VFs, or any combination of them, whose increased VF/ERF ratio(s) in a test sample relative to the VF/ERF ratio(s) in a control sample indicates the presence of Vascular Dementia, *i.e.*, VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, VF-372.

In a preferred embodiment, CSF from a subject is analyzed for quantitative detection of a plurality of VFs.

5.2 Vascular Dementia-Associated Protein Isoforms (VPIs)

In another aspect of the invention, CSF from a subject, preferably a living subject, is analyzed for quantitative detection of one or more Vascular Dementia-Associated Protein Isoforms (VPIs) for screening or diagnosis of Vascular Dementia, to determine the prognosis of a subject having Vascular Dementia, to monitor the effectiveness of Vascular Dementia therapy, for identifying patients most likely to respond to a particular therapeutic treatment or for drug development. As is well known in the art, a given protein may be expressed as variants (isoforms) that differ in their amino acid composition (*e.g.* as a result of alternative mRNA or premRNA processing, *e.g.* alternative splicing or limited proteolysis) or as a result

of differential post- translational modification (*e.g.*, glycosylation, phosphorylation, acylation), or both, so that proteins of identical amino acid sequence can differ in their pI, MW, or both. It follows that differential presence of a protein isoform does not require differential expression of the gene encoding the protein in question. As used herein, the term "Vascular Dementia-Associated Protein Isoform" refers to a protein isoform that is differentially present in CSF from a subject having Vascular Dementia compared with CSF from a subject free from Vascular Dementia. As used herein, the term "isoform" also refers to a protein that exists in only a single form, *i.e.*, it is not expressed as several variants.

Two groups of VPIs have been identified by amino acid sequencing of VFs. VPIs were isolated, subjected to proteolysis, and analyzed by mass spectrometry using the methods and apparatus of the Preferred Technology. One skilled in the art can identify sequence information from proteins analyzed by mass spectrometry and/or tandem mass spectrometry using various spectral interpretation methods and database searching tools. Examples of some of these methods and tools can be found at the Swiss Institute of Bioinformatics web site at http://www.expasy.ch/, and the European Molecular Biology Laboratory web site at www.mann.embl-heidelberg.de/Services/PeptideSearch/. Identification of VPIs was performed primarily using the SEQUEST search program (Eng et al, *J. Am. Soc. Mass Spectrom.* (1994) 5:976-989) with raw, uninterpreted tandem mass spectra of tryptic digest peptides as described in the Examples, *infra*. The first group consists of VPIs that are decreased in the CSF of subjects having Vascular Dementia as compared with the CSF of subjects free from Vascular Dementia, where the differential presence is significant. The amino acid sequences of tryptic digest peptides of these VPIs identified by tandem mass spectrometry and database searching as described in the Examples, *infra* are listed in Table IV in addition to the pIs and MWs of these VPIs.

Table IV. VPIs Decreased in CSF of Subjects Having Vascular Dementia

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-4	VPI-2	8.89	11749	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-5	VPI-3	9.16	20681	APEAQVSVQPNFQQDK, AQGFTEDTIVFLPQTDK
VF-12	VPI-6	7.62	70511	IPIEDGSGEVVLSR, TIYTPGSTVLYR, QELSEAEQATR
VF-13	VPI-7	6.18	105482	GCPTEEGCGER, AASGTQNNVLR

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-14	VPI-8	4.65	102603	HSIFTPETNPR; NLDENYCR, YEFLNGR, CEEDEEFTCR, EQQCVIMAENR
VF-15	VPI-90	5.73	14776	ASSIIDELFQDR
VF-16	VPI-9	4.65	32509	IPTTFENGR
VF-19	VPI-130	8.54	54625	VLLDGVQNPR, TIYTPGSTVLYR
VF-20	VPI-91	6.30	73287	DQYELLCR, FDQFFGEGCAPGSQR
VF-20	VPI-92	6.30	73287	GYTQQLAFR
VF-22	VPI-10	9.04	11790	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-25	VPI-131	9.32	13044	ALDFAVGEYNK
VF-26	VPI-11	9.18	48532	ALDFAVGEYNK, LVGGPMDASVEEEGVR
VF-27	VPI-93	9.76	13546	ALDFAVGEYNK, LVGGPMDASVEEEGVR
VF-29	VPI-13	5.26	11388	TMLLQPAGSLGSYSYR
VF-32	VPI-94	6.92	109447	WELCDIPR, CEEDEEFTCR
VF-32	VPI-95	6.92	109447	CFELQEAGPPDCR
VF-35	VPI-133	5.65	13084	AADDTWEPFASGK
VF-36	VPI-14	8.99	61111	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-37	VPI-15	9.80	23795	GFQALGDAADIR
VF-38	VPI-96	8.79	22458	TMLLQPAGSLGSYSYR
VF-41	VPI-17	4.91	38741	KGYTQQLAFR, AGDFLEANYMNLQR, DICEEQVNSLPGSITK, DFDFVPPVVR, GYTQQLAFR
VF-41	VPI-97	4.91	38741	ASSIIDELFQDR, ELDESLQVAER
VF-42	VPI-18	6.15	25232	TMLLQPAGSLGSYSYR, APEAQVSVQPNFQQDK
VF-43	VPI-19	5.67	48092	TVQAVLTVPK, ELLDTVTAPQK, SSFVAPLEK, LAAAVSNFGYDLYR, TSLEDFYLDEER, LSYEGEVTK, DTDTGALLFIGK
VF-43	VPI-98	5.67	48092	LCTVATLR

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-44	VPI-21	5.65	150534	VQVTSQEYSAR
VF-45	VPI-22	4.53	11037	GCSFLPDPYQK
VF-46	VPI-23	4.86	38741	AGDFLEANYMNLQR, DICEEQVNSLPGSITK, GYTQQLAFR, DFDFVPPVVR, KGYTQQLAFR, RQGALELIK
VF-47	VPI-24	5.49	57515	LPGIVAEGR
VF-47	VPI-99	5.49	57515	GSPAINVAVHVFR
VF-48	VPI-25	4.99	15072	GSPAINVAVHVFR
VF-50	VPI-27	5.48	55124	VLSALQAVQGLLVAQGR, DPTFIPAPIQAK, SLDFTELDVAAEK, ALQDQLVLVAAK
VF-50	VPI-28	5.48	55124	YTFELSR
VF-52	VPI-29	5.37	123390	AFLFQDTPR, LDQCYCER, NNAHGYFK, TCPTCNDFHGLVQK, TYFEGER, HNGQIWVLENDR, YLELESSGHR
VF-53	VPI-31	6.61	72071	TIYTPGSTVLYR, VMQDFFIDLR, QELSEAEQATR, IPIEDGSGEVVLSR
VF-54	VPI-100	4.57	13499	YLGYLEQLLR
VF-55	VPI-32	5.30	49423	LAAAVSNFGYDLYR, ALYYDLISSPDIHGTYK, SSFVAPLEK, ELLDTVTAPQK, DTDTGALLFIGK, LSYEGEVTK, TVQAVLTVPK, TSLEDFYLDEER
VF-55	VPI-33	5.30	49423	TALASGGVLDASGDYR
VF-57	VPI-37	9.18	39998	TLLSVGGWNFGSQR, FPLTNAIK, QHFTTLIK, FSNTDYAVGYMLR, GNQWVGYDDQESVK, EGDGSCFPDALDR, LVMGIPTFGR
VF-58	VPI-38	4.86	135312	AMQHISYLNSR, LICSELNGR, LTVTAYDCGK, YRPAEFHWK, EGLDLQVLEDSGR,

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
				GNLAGLTLR
VF-60	VPI-40	5.66	21021	GSIQVDGEELVSGR, GSIQVDGEDLVTGR, NLVLHSAR, LVSEDPINDGEWHR, YQLGSGEARGSGEAR, FSSGITGCVK, GSVYIGGAPDVATLTGGR
VF-64	VPI-43	5.77	52354	VLSALQAVQGLLVAQGR, DPTFIPAPIQAK, LQAILGVPWK, FMQAVTGWK, ALQDQLVLVAAK, SLDFTELDVAAEK
VF-66	VPI-46	5.01	25963	TMLLQPAGSLGSYSYR, APEAQVSVQPNFQQDK
VF-68	VPI-48	5.66	63184	ADLSGITGAR
VF-68	VPI-49	5.66	63184	FQNALLVR, KVPQVSTPTLVEVSR
VF-68	VPI-50	5.66	63184	GECQAEGVLFFQGDR, YYCFQGNQFLR, NFPSPVDAAFR, DYFMPCPGR, VWVYPPEK, RLWWLDLK
VF-171	VPI-158	8.40	14528	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-172	VPI-159	9.05	18350	APEAQVSVQPNFQQDK, TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK
VF-177	VPI-160	4.51	50790	VEQATQAIPMER, VIALINDQR
VF-180	VPI-161	9.12	38200	EGDGSCFPDALDR, TLLSVGGWNFGSQR, FSNTDYAVGYMLR, LVMGIPTFGR, GNQWVGYDDQESVK
VF-181	VPI-162	9.65	65654	SCGLHQLLR, VGDTLNLNLR
VF-188	VPI-163	7.89	29058	TGAQELLR
VF-189	VPI-164	8.54	34332	EELVYELNPLDHR, GLCVATPVQLR
VF-193	VPI-165	9.82	67749	LVGGPMDASVEEEGVR
VF-195	VPI-166	7.14	31448	CSVFYGAPSK, GLQDEDGYR, FACYYPR, VEYGFQVK, ITQVLHFTK
VF-203	VPI-167	4.12	93680	LLDSLPSDTR, FPVFMGR

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-204	VPI-168	7.13	32002	CSVFYGAPSK, GLQDEDGYR, VEYGFQVK, ITQVLHFTK
VF-207	VPI-169	8.01	34096	EPFLSCCQFAESLR, SFFPENWLWR, EFHLHLR, FEQLELR, GSFEFPVGDAVSK
VF-209	VPI-170	9.86	34695	FISLGEACK, VFLDCCNYITELR, TGLQEVEVK,
VF-209	VPI-171	9.86	34695	SVNDLYIQK, FPVEMTHNHNFR, TLEAQLTPR, NYNLVESLK
VF-210	VPI-172	4.67	65033	TATSEYQTFFNPR, ELLESYIDGR, SPQELLCGASLISDR
VF-212	VPI-173	9.39	11427	LVGGPMDASVEEEGVR
VF-213	VPI-174	5.29	54625	KFPSGTFEQVSQLVK, KLCMAALK, HLSLLTTLSNR, VCSQYAAYGEK, ELPEHTVK, LCDNLSTK, FEDCCQEK, YTFELSR, THLPEVFLSK,LPEATPTEL AK,
VF-213	VPI-175	5.29	54625	DPTFIPAPIQAK, VLSALQAVQGLLVAQGR, SLDFTELDVAAEK
VF-216	VPI-176	5.05	153158	EDYICYAR, IDGDTIIFSNVQER, QPEYAVVQR
VF-216	VPI-177	5.05	153158	QSEDSTFYLGER
VF-216	VPI-178	5.05	153158	WLQGSQELPR
VF-219	VPI-179	8.97	17091	LVGGPMDASVEEEGVR
VF-222	VPI-180	6.24	102603	AASGTQNNVLR, EQTMSECEAGALR
VF-223	VPI-181	7.89	57515	GDYPLEAVR, LFEELVR, GIFPVLCK, DPVQEAWAEDVDLR
VF-226	VPI-182	5.19	12080	GSPAINVAVHVFR
VF-227	VPI-183	5.04	40716	FSSCGGGGGSFGAGGGF GSR
VF-227	VPI-184	5.04	40716	LVPVVNNR
VF-227	VPI-185	5.04	40716	SGNENGEFYLR
VF-228	VPI-186	5.08	91613	GYHLNEEGTR

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-230	VPI-187	5.82	50026	YEAAVPDPR, EPGEFALLR, TALASGGVLDASGDYR
VF-232	VPI-188	8.16	34096	EPFLSCCQFAESLR, FEQLELR, GSFEFPVGDAVSK, EELVYELNPLDHR
VF-234	VPI-189	5.58	63762	GECQAEGVLFFQGDR, NFPSPVDAAFR, VWVYPPEK, DYFMPCPGR, RLWWLDLK
VF-234	VPI-190	5.58	63762	YLYEIAR, FQNALLVR, KVPQVSTPTLVEVSR,
VF-235	VPI-191	4.94	134070	GNLAGLTLR, EGLDLQVLEDSGR, QFPTPGIR
VF-237	VPI-192	4.93	102603	TGYYFDGISR, CLAFECPENYR, MCVDVNECQR
VF-239	VPI-193	4.26	75782	FEDGVLDPDYPR
VF-241	VPI-194	4.95	45574	TEQWSTLPPETK, DHAVDLIQK, ADGSYAAWLSR, VLSLAQEQVGGSPEK, AEMADQASAWLTR, QGSFQGGFR
VF-242	VPI-195	4.82	81483	VEQATQAIPMER, VIALINDQR
VF-243	VPI-196	4.31	88597	DFTCVHQALK, TLYSSSPR, LEDMEQALSPSVFK, FQPTLLTLPR
VF-245	VPI-197	6.18	187641	LPPNVVEESAR
VF-246	VPI-198	5.24	88817	TGYYFDGISR, CLAFECPENYR
VF-247	VPI-199	4.82	30552	FSSCGGGGGSFGAGGGF GSR
VF-249	VPI-200	5.10	27209	APEAQVSVQPNFQQDK
VF-250	VPI-201	6.00	49723	YEAAVPDPR, EPGEFALLR, TALASGGVLDASGDYR, VAMHLVCPSR, WVNLPEESLLR
VF-253	VPI-202	6.57	20336	EVDSGNDIYGNPIK, SDGSCAWYR
VF-254	VPI-203	4.46	32643	IPTTFENGR
VF-255	VPI-204	5.05	160613	QYTDSTFR
VF-256	VPI-205	6.77	34594	TSLEDFYLDEER

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-256	VPI-206	6.77	34594	NMQDMVEDYR
VF-258	VPI-207	5.19	91103	GYHLNEEGTR, TGYYFDGISR
VF-260	VPI-208	7.47	19095	LYTLVLTDPDAPSR,
VF-261	VPI-209	5.82	41902	TEDTIFLR
VF-261	VPI-210	5.82	41902	VNEPSILEMSR
VF-262	VPI-211	8.17	12814	LVGGPMDASVEEEGVR
VF-263	VPI-212	5.16	26050	THLAPYSDELR
VF-264	VPI-213	4.74	30882	IPTTFENGR

The second group comprises VPIs that are increased in the CSF of subjects having Vascular Dementia as compared with the CSF of subjects free from Vascular Dementia, where the differential presence is significant. The amino acid sequences of tryptic digest peptides of these VPIs identified by tandem mass spectrometry and database searching are listed in Table V in addition to the pIs and MWs of these VPIs.

Table V. VPIs Increased in CSF of Subjects Having Vascular Dementia

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-92	VPI-56	9.80	18109	RPASPISTIQPK, KLDGICWQVR, SLPVSDSVLSGFEQR, QLYGDTGVLGR
VF-92	VPI-57	9.80	18109	ALDFAVGEYNK, LVGGPMDASVEEEGVR
VF-93	VPI-146	9.59	18169	ISISTSGGSFR
VF-94	VPI-147	9.74	56994	VDFTLSSER, LNMGITDLQGLR, TTNIQGINLLFSSR
VF-95	VPI-108	9.47	37090	PYQYPALTPEQK
VF-97	VPI-58	7.24	11749	VVAGVANALAHK, GTFATLSELHCDK, LLVVYPWTQR
VF-98	VPI-61	7.48	11668	LVGGPMDASVEEEGVR
VF-98	VPI-109	7.48	11668	VHLTPEEK, VVAGVANALAHK, GTFATLSELHCDK, LLVVYPWTQR
VF-99	VPI-62	4.29	40288	WFYIASAFR, TEDTIFLR, EQLGEFYEALDCLR

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-100	VPI-63	7.50	55738	IPIEDGSGEVVLSR, QELSEAEQATR
VF-101	VPI-64	4.32	38629	TEDTIFLR, EQLGEFYEALDCLR
VF-102	VPI-65	5.91	64736	FQNALLVR, VPQVSTPTLVEVSR, LCTVATLR
VF-102	VPI-66	5.91	64736	NFPSPVDAAFR, DYFMPCPGR
VF-104	VPI-272	5.82	11533	AQGFTEDTIVFLPQTDK
VF-105	VPI-110	4.45	30662	NILTSNNIDVK, NPNLPPETVDSLK
VF-106	VPI-111	3.96	60192	EVEELMEDTQHK, DCQPGLCCAFQR, DQDGEILLPR
VF-107	VPI-273	6.31	17820	YTNWIQK
VF-108	VPI-148	7.88	11369	MFLSFPTTK
VF-109	VPI-112	6.18	15749	GLQDEDGYR
VF-112	VPI-149	6.81	57414	VGDTLNLNLR
VF-118	VPI-150	5.79	14996	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK
VF-122	VPI-67	7.44	26066	GGPLDGTYR, SADFTNFDPR
VF-122	VPI-68	7.44	26066	SGTASVVCLLNNFYPR, LLIYWASTR,
VF-123	VPI-152	6.65	13831	AQGFTEDTIVFLPQTDK, TMLLQPAGSLGSYSYR
VF-127	VPI-69	9.80	18843	TMLLQPAGSLGSYSYR
VF-127	VPI-70	9.80	18843	LVGGPMDASVEEEGVR
VF-135	VPI-269	6.45	20882	FSNTDYAVGYMLR, LVMGIPTFGR, GNQWVGYDDQESVK
VF-132	VPI-113	6.54	13783	LEEQAQQIR
VF-134	VPI-114	4.77	99610	TGYYFDGISR
VF-136	VPI-71	9.58	20268	TMLLQPAGSLGSYSYR
VF-137	VPI-270	7.26	12594	LYTLVLTDPDAPSR, YVWLVYEQDR
VF-138	VPI-72	9.22	16179	APEAQVSVQPNFQQDK, TMLLQPAGSLGSYSYR
VF-139	VPI-73	9.22	19032	AQGFTEDTIVFLPQTDK, TMLLQPAGSLGSYSYR
VF-140	VPI-271	6.11	31600	SSFVAPLEK,

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
				TSLEDFYLDEER
VF-141	VPI-75	4.40	146682	FFEECDPNK, AQSIAYHLK
VF-142	VPI-77	6.35	19414	SDGSCAWYR, EVDSGNDIYGNPIK
VF-143	VPI-78	9.58	21021	TMLLQPAGSLGSYSYR
VF-144	VPI-79	7.42	56136	VLLDGVQNPR, IPIEDGSGEVVLSR, TIYTPGSTVLYR, QELSEAEQATR, GLEVTITAR
VF-145	VPI-80	8.16	59646	EYESYSDFER, CEGFVCAQTGR
VF-147	VPI-115	6.23	12206	EGIPPDQQR
VF-147	VPI-116	6.23	12206	FEETTADGR
VF-148	VPI-117	5.66	146050	ALEESNYELEGK
VF-149	VPI-81	6.56	20744	EVDSGNDIYGNPIK, SDGSCAWYR,
VF-150	VPI-118	7.48	59646	LNMGITDLQGLR, GQIVFMNR, EMSGSPASGIPVK
VF-151	VPI-82	7.47	22090	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK
VF-152	VPI-153	6.86	50636	FQNALLVR
VF-153	VPI-83	6.74	54791	QELSEAEQATR, VLLDGVQNPR, TIYTPGSTVLYR
VF-153	VPI-119	6.74	54791	ATVVYQGER
VF-154	VPI-84	7.27	94587	QELSEAEQATR, IPIEDGSGEVVLSR, TIYTPGSTVLYR
VF-155	VPI-120	6.13	88018	QDACQGDSGGVFAVR
VF-156	VPI-85	5.97	14520	QPVPGQQMTLK, IWDVVEK
VF-157	VPI-121	9.24	21021	AQGFTEDTIVFLPQTDK, TMLLQPAGSLGSYSYR, APEAQVSVQPNFQQDK
VF-158	VPI-122	9.26	21908	AQGFTEDTIVFLPQTDK, TMLLQPAGSLGSYSYR
VF-159	VPI-123	9.78	29583	ETAASLLQAGYK, SPQELLCGASLISDR
VF-160	VPI-124	6.21	67544	DGFVQDEGTMFPVGK
VF-161	VPI-268	6.11	74524	HVVPNEVVVQR, AGALNSNDAFVLK,

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
				TGAQELLR
VF-163	VPI-87	6.53	10226	SCDLALLETYCATPAK, GIVEECCFR
VF-164	VPI-125	8.44	19222	AQGFTEDTIVFLPQTDK
VF-166	VPI-154	5.02	67749	QDIVFDGIAQIR, AFQVWSDVTPLR
VF-166	VPI-155	5.02	67749	DGNTLTYYR, AIEDYINEFSVR
VF-166	VPI-156	5.02	67749	WEDILSDEVNVAR, GVALADFNR
VF-168	VPI-88	5.22	13359	GSPAINVAVHVF AADDTWEPFASGK,
VF-170	VPI-127	4.62	28747	ASSIIDELFQDR
VF-170	VPI-128	4.62	28747	NPNLPPETVDSLK, NILTSNNIDVK, IPTTFENGR
VF-170	VPI-129	4.62	28747	GECQAEGVLFFQGDR
VF-275	VPI-214	4.23	39766	WFYIASAFR, TEDTIFLR, YVGGQEHFAHLLILR, TYMLAFDVNDEK, NWGLSVYADKPETTK, EQLGEFYEALDCLR, SDVVYTDWK
VF-277	VPI-215	4.24	41761	WFYIASAFR, TEDTIFLR, YVGGQEHFAHLLILR, TYMLAFDVNDEK, NWGLSVYADKPETTK, EQLGEFYEALDCLR, SDVVYTDWK
VF-280	VPI-216	9.31	11137	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-281	VPI-217	10.06	11587	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-282	VPI-218	5.98	45728	YICENQDSISSK, DVFLGMFLYEYAR, VPQVSTPTLVEVSR
VF-287	VPI-219	7.83	23298	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK,
VF-290	VPI-220	9.81	10923	LVGGPMDASVEEEGVR
VF-291	VPI-221	9.91	27009	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-292	VPI-222	4.83	56582	ADQVCINLR, SGNENGEFYLR
VF-295	VPI-223	4.39	30225	IPTTFENGR
VF-299	VPI-224	5.49	158545	GAYPLSIEPIGVR,

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
				NNEGTYYSPNYNPQSR, QSEDSTFYLGER
VF-302	VPI-225	7.65	61670	ADSGEGDFLAEGGGVR, GGSTSYGTGSETESPR
VF-303	VPI-226	9.83	39766	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-305	VPI-227	4.69	53640	HTLNQIDEVK
VF-306	VPI-228	5.85	45882	YICENQDSISSK, CCAAADPHECYAK, FQNALLVR, VPQVSTPTLVEVSR, AVMDDFAAFVEK
VF-309	VPI-229	6.03	13175	GSPAINVAVHVFR
VF-311	VPI-230	6.15	45719	CCAAADPHECYAK, FQNALLVR, VPQVSTPTLVEVSR
VF-313	VPI-231	4.53	30225	IPTTFENGR
VF-314	VPI-232	6.98	59466	AEFQDALEK, LNMGITDLQGLR, VGDTLNLNLR
VF-316	VPI-233	6.08	30920	TSLEDFYLDEER
VF-319	VPI-234	7.38	59828	HVVPNEVVVQR
VF-324	VPI-235	6.39	44664	IVQLIQDTR, SIPQVSPVR
VF-324	VPI-236	6.39	44664	LVAEFDFR
VF-329	VPI-237	9.05	19478	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK, APEAQVSVQPNFQQDK
VF-330	VPI-238	4.77	18049	MFGRPWSR
VF-331	VPI-239	6.28	67135	EQTMSECEAGALR,
VF-332	VPI-240	6.22	23973	LVNEVTEFAK, AAFTECCQAADK, SLHTLFGDK
VF-333	VPI-241	7.14	32549	VHYTVCIWR, CSVFYGAPSK, GLQDEDGYR, FACYYPR, VEYGFQVK, ITQVLHFTK
VF-333	VPI-242	7.14	32549	TSLEDFYLDEER
VF-334	VPI-243	6.06	11270	VNHVTLSQPK
VF-335	VPI-244	6.70	38112	VPTVDVSVVDLTVR,
VF-336	VPI-245	5.75	24567	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK,
VF-337	VPI-246	6.73	62107	IPSETLNR
VF-340	VPI-247	6.07	65130	GECQAEGVLFFQGDR,

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
				NFPSPVDAAFR
VF-341	VPI-248	7.01	40510	LNDLEEALQQAK
VF-342	VPI-249	8.16	24182	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK, APEAQVSVQPNFQQDK
VF-343	VPI-250	5.47	51028	TSLEDFYLDEER, LAAAVSNFGYDLYR, SSFVAPLEK
VF-344	VPI-251	6.66	19935	ALEESNYELEGK
VF-346	VPI-252	5.73	22738	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK
VF-346	VPI-253	5.73	22738	LVNEVTEFAK, YLYEIAR
VF-348	VPI-254	5.10	29902	TGAQELLR
VF-349	VPI-255	6.35	53084	DFYVDENTTVR
VF-351	VPI-256	9.07	23405	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK
VF-352	VPI-257	6.57	19011	EVDSGNDIYGNPIK
VF-353	VPI-258	6.69	39193	INHGILYDEEK, EIMENYNIALR
VF-356	VPI-259	5.44	53312	YTFELSR
VF-356	VPI-260	5.44	53312	DPTFIPAPIQAK, VLSALQAVQGLLVAQGR
VF-359	VPI-261	5.02	80131	HQFLLTGDTQGR, CEGPIPDVTFELLR
VF-360	VPI-262	4.57	30225	IPTTFENGR
VF-361	VPI-263	4.55	24374	TMLLQPAGSLGSYSYR
VF-362	VPI-264	7.29	20444	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK, APEAQVSVQPNFQQDK
VF-365	VPI-265	4.41	24762	LPYTASSGLMAPR
VF-368	VPI-266	5.14	13240	LVNEVTEFAK
VF-370	VPI-267	4.40	27223	IPTTFENGR

As will be evident to one of skill in the art, based upon the present description, a given VPI can be described according to the data provided for that VPI in Table IV or V. The VPI is a protein comprising a peptide sequence described for that VPI (preferably comprising a plurality of, more preferably all of, the peptide sequences described for that VPI) and has a pI of about the value stated for that VPI (preferably within 10%, more preferably within 5% still more preferably within 1% of the stated value) and has a MW of about the value stated for that VPI (preferably within 10%, more preferably within 5%, still more preferably within 1% of

the stated value). Proteins comprising the peptide sequences provided in Table IV and V can be identified by searching sequence databases with those peptides using search tools known to those skilled in the art. Examples of search algorithm tools that can be used to identify proteins from peptide sequences include:

- BLAST (Basic Local Alignment Search Tool) : BLAST is maintained at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and is based on a statistical theory developed by Samuel Karlin and Steven Altschul (*Proc. Natl Acad. Sci.* USA (1990) 87:2284-2268), later modified as in Karlin and Altschul (*Proc. Natl Acad. Sci.* (1993) 90:5873). BLASTP can be used to search a protein sequence against a protein database. TBLASTN can be used to search a Protein Sequence against a Nucleotide Database, by translating each database Nucleotide sequence in all 6 reading frames.
- FASTA as described in Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-8. See also Pearson *Methods Enzymol.* (1990) 183:63-98 and Pearson *Genomics* (1991) 11(3):635-50.

Examples of available protein sequence databases include:

- The nr protein database maintained at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The nr protein database is compiled of entries from various sources including SwissProt, SwissProt updates, PIR, and PDB. The BLAST resource is available for sequence searching.
- SwissProt and TrEMBL databases developed by the Swiss Bioinformatics Institute (SIB) and the European can be found at http://www.expasy.ch. BLASTP resources are available for sequence searching.
- The PIR-International Protein Sequence Database maintained by the Protein Information Resource (PIR), in collaboration with the Munich Information Center for Protein Sequences (MIPS) and the Japanese International Protein Sequence Database (JIPID). The Protein Identification Resource (PIR) is a division of the National Biomedical Research Foundation (NBRF) which is affiliated with Georgetown University Medical Center and can be found at http://www nbrf.georgetown.edu/pir/searchdb.html. The database can be searched using BLAST and FASTA search algorithm tools.
- The Protein Data Bank, maintained by Brookhaven National Laboratory (Long Island, New York, USA) which can be found at http://www.rcsb.org/pdb/. The FASTA resource is available at this website for sequence searching.

In one embodiment, CSF from a subject is analyzed for quantitative detection of one or more of the following VPIs: VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-130, VPI-131, VPI-133, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213, or any combination of them, wherein a decreased abundance of the VPI or VPIs (or any combination of them) in the CSF from the subject relative to CSF from a subject or subjects free from Vascular Dementia (*e.g.*, a control sample or a previously determined reference range) indicates the presence of Vascular Dementia.

In another embodiment of the invention, CSF from a subject is analyzed for quantitative detection of one or more of the following VPIs: VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246, VPI-247, VPI-248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, VPI-273, or any combination of them, wherein an increased abundance of the VPI or VPIs (or any combination of them) in CSF from the subject relative to CSF from a subject or subjects free from Vascular Dementia (*e.g.*, a control sample or a previously determined reference range) indicates the presence of Vascular Dementia.

In a further embodiment, CSF from a subject is analyzed for quantitative detection of (a) one or more VPIs, or any combination of them, whose decreased abundance indicates the presence of Vascular Dementia, *i.e.*, VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-130, VPI-131, VPI-133, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213; and (b) one or more VPIs, or any combination of them, whose increased abundance indicates the presence of Vascular Dementia, *i.e.*, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246, VPI-247, VPI-248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, VPI-273.

In yet a further embodiment, CSF from a subject is analyzed for quantitative detection of one or more VPIs and one or more previously known biomarkers of Vascular Dementia (*e.g.*, candidate markers such as hypersensitive platelet glutamate receptors (Berk et al, *Int Clin Psychopharmacol* (1999) 14:199-122)). In accordance with this embodiment, the abundance of each VPI and known biomarker relative to a control or reference range indicates whether a subject has Vascular Dementia.

Preferably, the abundance of a VPI is normalized to an Expression Reference Protein Isoform (ERPI). ERPIs can be identified by partial amino acid sequencing of ERFs, which are described above, using the methods and apparatus of the Preferred Technology. The partial amino acid sequences of an ERPI, and the known proteins to which it is homologous is presented in Table VI.

Table VI. Expression Reference Protein Isoforms

ERF#	ERPI #	Amino Acid Sequences of Tryptic Digest Peptides
ERF-1	ERPI-1	GLQDEDGYR
ERF-2	ERPI-2	DPTFIPAPIQAK, ALQDQLVLVAAK

As shown above, the VPIs described herein include previously unknown proteins, as well as isoforms of known proteins where the isoforms were not previously known to be associated with Vascular Dementia. For each VPI, the present invention additionally provides: (a) a preparation comprising the isolated VPI; (b) a preparation comprising one or more fragments of the VPI; and (c) antibodies that bind to said VPI, to said fragments, or both to said VPI and to said fragments. As used herein, a VPI is "isolated" when it is present in a preparation that is substantially free of contaminating proteins, *i.e.*, a preparation in which less than 10% (preferably less than 5%, more preferably less than 1%) of the total protein present is contaminating protein(s). A contaminating protein is a protein or protein isoform having a significantly different pI or MW from those of the isolated VPI, as determined by 2D electrophoresis. As used herein, a "significantly different" pI or MW is one that permits the contaminating protein to be resolved from the VPI on 2D electrophoresis, performed according to the Reference Protocol.

In one embodiment, an isolated protein is provided, said protein comprising a peptide with the amino acid sequence identified in Table IV or V for a VPI, said protein having a pI and MW within 10% (preferably within 5%, more preferably within 1%) of the values identified in Table IV or V for that VPI.

The VPIs of the invention can be qualitatively or quantitatively detected by any method known to those skilled in the art, including but not limited to the Preferred Technology described herein, kinase assays, enzyme assays, binding assays and other functional assays, immunoassays, and western blotting. In one embodiment, the VPIs are separated on a 2-D gel

by virtue of their MWs and pIs and visualized by staining the gel. In one embodiment, the VPIs are stained with a fluorescent dye and imaged with a fluorescence scanner. Sypro Red (Molecular Probes, Inc., Eugene, Oregon) is a suitable dye for this purpose. A preferred fluorescent dye is Pyridinium, 4-[2-[4- (dipentylamino)-2-trifluoromethylphenyl] ethenyl]-1-(sulfobutyl)-, inner salt. See U.S. Application No. 09/412,168, filed on October 5, 1999, which is incorporated herein by reference in its entirety.

Alternatively, VPIs can be detected in an immunoassay. In one embodiment, an immunoassay is performed by contacting a sample from a subject to be tested with an anti-VPI antibody under conditions such that immunospecific binding can occur if the VPI is present, and detecting or measuring the amount of any immunospecific binding by the antibody. Anti-VPI antibodies can be produced by the methods and techniques taught herein; examples of such antibodies known in the art are set forth in Table VII. These antibodies shown in Table VII are already known to bind to the protein of which the VPI is itself a family member. Preferably, the anti-VPI antibody preferentially binds to the VPI rather than to other isoforms of the same protein. In a preferred embodiment, the anti-VPI antibody binds to the VPI with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other isoforms of the same protein.

VPIs can be transferred from the gel to a suitable membrane (*e.g.* a PVDF membrane) and subsequently probed in suitable assays that include, without limitation, competitive and non-competitive assay systems using techniques such as western blots and "sandwich" immunoassays using anti-VPI antibodies as described herein, *e.g.*, the antibodies identified in Table VII, or others raised against the VPIs of interest. The immunoblots can be used to identify those anti-VPI antibodies displaying the selectivity required to immuno-specifically differentiate a VPI from other isoforms encoded by the same gene.

Table VII. Known Antibodies That Recognize VPIs or VPI-Related Polypeptides

VPI#	Antibody	Manufacturer	Cat. No.
VPI-2	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-6	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-7	C7 Complement, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- G34
VPI-8	Monoclonal mouse anti-human plasminogen	RDI RESEARCH DIAGNOSTICS, INC	RDI-TRK4P11-4D2

VPI#	Antibody	Manufacturer	Cat. No.
VPI-9	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-10	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-11	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-14	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-15	Tissue Inhibitor of Matrix Metalloproteinase 1 (TIMP1) (NO X w/TIMP2), Clone: 2A5, Mab anti-Human, paraffin, IH	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA497
VPI-17	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-23	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-24	Antithrombin III, Clone: BL-ATIII/3, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 9009-1
VPI-25	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-27	AT1 (306)	SANTA CRUZ BIOTECHNOLOGY, INC - RESEARCH ANTIBODIES 98/99	sc-579
VPI-31	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-43	AT1 (306)	SANTA CRUZ BIOTECHNOLOGY, INC - RESEARCH ANTIBODIES 98/99	sc-579
VPI-48	Alpha-1-Antichymotrypsin, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 145/2
VPI-49	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-50	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
VPI-56	C8 Complement, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- G35
VPI-57	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL &	AXL- 574

VPI#	Antibody	Manufacturer	Cat. No.
		SCIENTIFIC CORPORATION	
VPI-58	Hemoglobin, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- J16
VPI-61	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-62	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-63	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-64	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-65	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-66	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
VPI-67	Kappa Chain, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- 021D
VPI-70	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-77	Tissue Inhibitor of Matrix Metalloproteinase 2 (TIMP2) (NO X w/TIMP1), Clone: 3A4, Mab anti-Human, paraffin, IH	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA498
VPI-79	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-80	C8 Complement, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- G35
VPI-81	Tissue Inhibitor of Matrix Metalloproteinase 2 (TIMP2) (NO X w/TIMP1), Clone: 3A4, Mab anti-Human, paraffin, IH	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA498
VPI-83	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-84	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-87	Insulin Like Growth Factor II (IGF-II), Clone: W2H1, Mab anti-, frozen, IH/ELISA/RIA	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MAS- 976p

VPI#	Antibody	Manufacturer	Cat. No.
VPI-88	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-90	Goat anti-Clusterin (human)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CLUSTRCabG
VPI-91	Monoclonal mouse anti-lactoferrin	RDI RESEARCH DIAGNOSTICS, INC	RDI-TRK4L2-LF2B8
VPI-92	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-93	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-94	Monoclonal mouse anti-human plasminogen	RDI RESEARCH DIAGNOSTICS, INC	RDI-TRK4P11-4D2
VPI-97	Goat anti-Clusterin (human)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CLUSTRCabG
VPI-98	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-99	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-109	Hemoglobin, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- J16
VPI-110	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-112	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-113	Apolipoprotein E, LDL, VLDL, Clone: 3D12, Mab anti-Human, frozen/paraffin	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YM- 5029
VPI-117	ANTI-CYTOKERATIN TYPE 10	RDI RESEARCH DIAGNOSTICS, INC	RDI-CBL196
VPI-118	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-120	C1r Complement, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YSRT- AHC002
VPI-123	Prothrombin, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 448/2
VPI-127	Goat anti-Clusterin (human)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CLUSTRCabG
VPI-128	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457

VPI#	Antibody	Manufacturer	Cat. No.
VPI-129	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
VPI-130	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-131	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-133	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-147	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-149	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-153	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-158	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-162	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-163	Gelsolin, plasma + cytoplasmic, Sheep anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YBG- 4628-6210
VPI-164	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-165	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-166	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-168	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-169	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-170	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-171	Heparin Cofactor II, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHCFII
VPI-172	Prothrombin, Rabbit anti-Human	ACCURATE CHEMICAL &	AXL- 448/2

VPI#	Antibody	Manufacturer	Cat. No.
		SCIENTIFIC CORPORATION	
VPI-173	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-175	AT1 (306)	SANTA CRUZ BIOTECHNOLOGY, INC - RESEARCH ANTIBODIES 98/99	sc-579
VPI-178	Monoclonal mouse anti-human IgA1	RDI RESEARCH DIAGNOSTICS, INC	RDI-TRK1A2-2B5
VPI-179	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-180	C7 Complement, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- G34
VPI-182	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-183	Polylconal Rabbit anti-Human Cytokeratin 1 (Keratin 1)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CYTOK1abr
VPI-188	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-189	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
VPI-190	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-194	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-199	Polylconal Rabbit anti-Human Cytokeratin 1 (Keratin 1)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CYTOK1abr
VPI-202	Tissue Inhibitor of Matrix Metalloproteinase 2 (TIMP2) (NO X w/TIMP1), Clone: 3A4, Mab anti-Human, paraffin, IH	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA498
VPI-203	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-206	Polylconal Rabbit anti-Human Cytokeratin 1 (Keratin 1)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CYTOK1abr
VPI-209	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-211	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574

VPI#	Antibody	Manufacturer	Cat. No.
VPI-212	Apolipoprotein A (HDL), Sheep anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	ACL- 20075AP
VPI-213	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-214	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-215	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-216	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-217	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-218	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-220	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-221	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-223	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-226	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-228	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-229	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-230	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-231	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-232	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-234	Gelsolin, plasma + cytoplasmic, Sheep anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YBG- 4628-6210
VPI-239	C7 Complement, Goat anti-	ACCURATE CHEMICAL &	BMD- G34

VPI#	Antibody	Manufacturer	Cat. No.
	Human	SCIENTIFIC CORPORATION	
VPI-240	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-241	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-247	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
VPI-251	ANTI-CYTOKERATIN TYPE 10	RDI RESEARCH DIAGNOSTICS, INC	RDI-CBL196
VPI-253	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-254	Gelsolin, plasma + cytoplasmic, Sheep anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YBG- 4628-6210
VPI-255	Monoclonal anti-Prekallikrein Heavy Chain	BIODESIGN INTERNATIONAL	N55199M
VPI-257	Tissue Inhibitor of Matrix Metalloproteinase 2 (TIMP2) (NO X w/TIMP1), Clone: 3A4, Mab anti-Human, paraffin, IH	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA498
VPI-258	Factor H (Complement), Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-066-02
VPI-260	AT1 (306)	SANTA CRUZ BIOTECHNOLOGY, INC - RESEARCH ANTIBODIES 98/99	sc-579
VPI-261	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-262	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-266	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-267	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-268	Gelsolin, plasma + cytoplasmic, Sheep anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YBG- 4628-6210

*Further information about these antibodies can be obtained from their commercial sources at: ACCURATE CHEMICAL & SCIENTIFIC CORPORATION http://www.accuratechemical.com; BIODESIGN INTERNATIONAL -http://www.biodesign.com/; RDI RESEARCH DIAGNOSTICS, INC - http://www.researchd.com/

5

In one embodiment, binding of antibody in tissue sections can be used to detect aberrant VPI localization or an aberrant level of one or more VPIs. In a specific embodiment, antibody to a VPI can be used to assay a tissue sample (*e.g.*, a brain biopsy) from a subject for the level of the VPI where an aberrant level of VPI is indicative of Vascular Dementia. As used herein, an "aberrant level" means a level that is increased or decreased compared with the level in a subject free from Vascular Dementia or a reference level. If desired, the comparison can be performed with a matched sample from the same subject, taken from a portion of the body not affected by Vascular Dementia.

Any suitable immunoassay can be used, including, without limitation, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement- fixation assays, immunoradiometric assays, fluorescent immunoassays and protein A immunoassays.

For example, a VPI can be detected in a fluid sample (*e.g.*, CSF, blood, urine, or tissue homogenate) by means of a two-step sandwich assay. In the first step, a capture reagent (*e.g.*, an anti-VPI antibody) is used to capture the VPI. Examples of such antibodies known in the art are set forth in Table VII. The capture reagent can optionally be immobilized on a solid phase. In the second step, a directly or indirectly labelled detection reagent is used to detect the captured VPI. In one embodiment, the detection reagent is a lectin. Any lectin can be used for this purpose that preferentially binds to the VPI rather than to other isoforms that have the same core protein as the VPI or to other proteins that share the antigenic determinant recognized by the antibody. In a preferred embodiment, the chosen lectin binds to the VPI with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other isoforms that have the same core protein as the VPI or to said other proteins that share the antigenic determinant recognized by the antibody. Based on the present description, a lectin that is suitable for detecting a given VPI can readily be identified by methods well known in the art, for instance upon testing one or more lectins enumerated in Table I on pages 158-159 of Sumar et al, Lectins as Indicators of Disease-Associated Glycoforms, In: Gabius H-J & Gabius S (eds.), 1993, Lectins and Glycobiology, at pp. 158-174 (which is incorporated herein by reference in its entirety). Lectins with the desired oligosaccharide specificity can be identified, for example, by their ability to detect the VPI in a 2D gel, in a replica of a 2D gel following transfer to a suitable solid substrate such as a nitrocellulose membrane, or in a two-step assay following capture by

an antibody. In an alternative embodiment, the detection reagent is an antibody, *e.g.*, an antibody that immunospecifically detects other post-translational modifications, such as an antibody that immunospecifically binds to phosphorylated amino acids. Examples of such antibodies include those that bind to phosphotyrosine (BD Transduction Laboratories, catalog nos.: P11230-050/P11230-150; P11120; P38820; P39020), those that bind to phosphoserine (Zymed Laboratories Inc., South San Francisco, CA, catalog no. 61- 8100) and those that bind to phosphothreonine (Zymed Laboratories Inc., South San Francisco, CA, catalog nos. 71-8200, 13-9200).

If desired, a gene encoding a VPI, a related gene, or related nucleic acid sequences or subsequences, including complementary sequences, can also be used in hybridization assays. A nucleotide encoding a VPI, or subsequences thereof comprising at least 8 nucleotides, preferably at least 12 nucleotides, and most preferably at least 15 nucleotides can be used as a hybridization probe. Hybridization assays can be used for detection, prognosis, diagnosis, or monitoring of conditions, disorders, or disease states, associated with aberrant expression of genes encoding VPIs, or for differential diagnosis of subjects with signs or symptoms suggestive of Vascular Dementia. In particular, such a hybridization assay can be carried out by a method comprising contacting a subject's sample containing nucleic acid with a nucleic acid probe capable of hybridizing to a DNA or RNA that encodes a VPI, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization. Nucleotides can be used for therapy of subjects having Vascular Dementia, as described below.

The methods and compositions for clinical screening, diagnosis and prognosis of Vascular Dementia in a mammalian subject may be diagnostic of Vascular Dementia or indicative of Vascular Dementia.

Diagnostic methods and compositions are based on Vascular Dementia-Associated Features (VFs) and Vascular Dementia-Associated Protein Isoforms (VPIs) which are specifically and particularly associated with Vascular Dementia and are generally not associated with other diseases or conditions. Such diagnostic VFs or VPIs, which are specifically associated with Vascular Dementia, are useful in screening, diagnosis and prognosis as indicators of Vascular Dementia. The administration of therapeutic compositions which are directed against or lead to modulation of diagnostic markers may have therapeutic value particularly in Vascular Dementia.

Indicative methods and compositions are based on Vascular Dementia-Associated Features (VFs) and Vascular Dementia-Associated Protein Isoforms (VPIs) which are

associated with Vascular Dementia but may not be specific only for Vascular Dementia, and may be associated with one or more other diseases or conditions. Such indicative VFs or VPIs, which are associated with Vascular Dementia, but not only with Vascular Dementia, are useful in screening, diagnosis and prognosis as indicators of Vascular Dementia. Indicative methods and compositions are particularly useful in the initial or general screening, diagnosis and prognosis of an individual subject, whereby a first indication of a subset of conditions or diseases, including Vascular Dementia, is thereby provided. Additional assessment utilizing diagnostic or particular Vascular Dementia VFs or VPIs may then be undertaken to provide specific, diagnostic screening, diagnosis and prognosis of the individual subject. The administration of therapeutic compositions which are directed against or lead to modulation of indicative markers may have therapeutic value in Vascular Dementia and other disorders as well, or may be useful therapeutically in more than one disease or condition

Thus, a diagnostic marker changes (increases, decreases or otherwise alters form or character) significantly in only a single disease or condition or in only a small number of conditions, particularly in related conditions. Two such diagnostic markers, VF-37 and VF-50, are provided below in Table VIII.

Table VIII. Example of a diagnostic marker for Vascular Dementia:

Feature #	Isoform #	Fold Change	pI	MW (Da)
VF-50	VPI-27	-2.43	5.48	55124

An indicative marker changes (increases, decreases or otherwise alters form or character) significantly in more than one condition, particularly in Vascular Dementia and one or more other distinct diseases or conditions. One such indicative marker, VF-149, is found to increase in Vascular Dementia and is provided in Table IX. This same marker, identified or characterised by the same pI and MW, is noted as SF-219 as similarly found to be increased in Schizophrenia. The VF-149/SF-219 marker is therefore indicative of Vascular Dementia and/or Schizophrenia.

Table IX: Example of an Indicative Marker for Vascular Dementia:

Feature #	Isoform #	Disease	Fold Change	pI	MW (Da)
VF-149	VPI-81	Vascular Dementia	3.18	6.56	20744
SF-219	SPI-114	Schizophrenia	2.77	6.56	20744

The invention also provides diagnostic kits, comprising an anti-VPI antibody. In addition, such a kit may optionally comprise one or more of the following: (1) instructions for using the anti-VPI antibody for diagnosis, prognosis, therapeutic monitoring or any combination of these applications; (2) a labelled binding partner to the antibody; (3) a solid phase (such as a reagent strip) upon which the anti-VPI antibody is immobilized; and (4) a label or insert indicating regulatory approval for diagnostic, prognostic or therapeutic use or any combination thereof. If no labelled binding partner to the antibody is provided, the anti-VPI antibody itself can be labelled with a detectable marker, *e.g.*, a chemiluminescent, enzymatic, fluorescent, or radioactive moiety.

The invention also provides a kit comprising a nucleic acid probe capable of hybridizing to RNA encoding a VPI. In a specific embodiment, a kit comprises in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides, more preferably 10-30 nucleotides and still more preferably 10-20 nucleotides) that under appropriate reaction conditions can prime amplification of at least a portion of a nucleic acid encoding a VPI, such as by polymerase chain reaction (see, *e.g.*, Innis et al, 1990, PCR Protocols, Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of Qβ replicase, cyclic probe reaction, or other methods known in the art.

Kits are also provided which allow for the detection of a plurality of VPIs or a plurality of nucleic acids each encoding a VPI. A kit can optionally further comprise a predetermined amount of an isolated VPI protein or a nucleic acid encoding a VPI, *e.g.*, for use as a standard or control.

5.3 Statistical Techniques for Identifying VPIs and VPI Clusters

The uni-variate differential analysis tools, such as fold changes, wilcoxon rank sum test and t-test, are useful in identifying individual VFs or VPIs that are diagnostically associated with Vascular Dementia or in identifying individual VPIs that regulate the disease process. In most cases, however, those skilled in the art appreciate that the disease process is associated with a combination of VFs or VPIs (and to be regulated by a combination of VPIs), rather than individual VFs and VPIs in isolation. The strategies for discovering such combinations of VFs and VPIs differ from those for discovering individual VFs and VPIs. In such cases, each individual VF and VPI can be regarded as one variable and the disease can be regarded as a joint, multi-variate effect caused by interaction of these variables.

The following steps can be used to identify markers from data produced by the Preferred Technology.

The first step is to identify a collection of VFs or VPIs that individually show significant association with Vascular Dementia. The association between the identified VFs or VPIs and Vascular Dementia need not be as highly significant as is desirable when an individual VF or VPI is used as a diagnostic. Any of the tests discussed above (fold changes, wilcoxon rank sum test, etc.) can be used at this stage. Once a suitable collection of VFs or VPIs has been identified, a sophisticated multi-variate analysis capable of identifying clusters can then be used to estimate the significant multivariate associations with Vascular Dementia.

Linear Discriminant Analysis (LDA) is one such procedure, which can be used to detect significant association between a cluster of variables (*i.e.*, VFs or VPIs) and Vascular Dementia. In performing LDA, a set of weights is associated with each variable (*i.e.*, VF or VPI) so that the linear combination of weights and the measured values of the variables can identify the disease state by discriminating between subjects having Vascular Dementia and subjects free from Vascular Dementia. Enhancements to the LDA allow stepwise inclusion (or removal) of variables to optimize the discriminant power of the model. The result of the LDA is therefore a cluster of VFs or VPIs which can be used, without limitation, for diagnosis, prognosis, therapy or drug development. Other enhanced variations of LDA, such as Flexible Discriminant Analysis permit the use of non-linear combinations of variables to discriminate a disease state from a normal state. The results of the discriminant analysis can be verified by post-hoc tests and also by repeating the analysis using alternative techniques such as classification trees.

A further category of VFs or VPIs can be identified by qualitative measures by comparing the percentage feature presence of a VF or VPI of one group of samples (*e.g.*, samples from diseased subjects) with the percentage feature presence of a VF or VPI in another group of samples (*e.g.*, samples from control subjects). The "percentage feature presence" of a VF or VPI is the percentage of samples in a group of samples in which the VF or VPI is detectable by the detection method of choice. For example, if a VF is detectable in 95 percent of samples from diseased subjects, the percentage feature presence of that VF in that sample group is 95 percent. If only 5 percent of samples from non-diseased subjects have detectable levels of the same VF, detection of that VF in the sample of a subject would suggest that it is likely that the subject suffers from Vascular Dementia.

5.4 Use in Clinical Studies

The diagnostic methods and compositions of the present invention can assist in monitoring a clinical study, *e.g.* to evaluate drugs for therapy of Vascular Dementia. In one

embodiment, candidate molecules are tested for their ability to restore VF or VPI levels in a subject having Vascular Dementia to levels found in subjects free from Vascular Dementia or, in a treated subject (*e.g.* after treatment with antiplatelet agents such as aspirin, Buflomedil (Cucinotta et al. *J Int Med Res* (1992) 20:136-49), neuroprotective agents such as Propentofylline (Rother et al. Ann *N Y Acad Sci* (1996) 777:404-9, Mielke et al. *Alzheimer Dis Assoc Disord* (1998) 12 Suppl 2:S29-35, Rother et al. *Dement Geriatr Cogn Disord* (1998) 9 Suppl 1:36-43), cholinesterase inhibitors such as rivastigmine, galantamine (Kumar et al. *Neurology* (1999) 52 Suppl 2:A395) and other cytoprotective agents currently under clinical evaluation such as the calcium antagonists Nimodipine and Nicadipine, NMDA antagonists such as Selfotel, Dextrorphan, Cerestat, Eliprodil, Lamortigine, GABA agonists, Kappa-selective opiod antagonists, Lubeluzole, Free radicalscavengers, anti-ICAM antibodies and GM-1 ganglioside, Abbokinase®, Activase®,Aggrenox®, Anti-ICAM-1 antibody, Anti-beta-2-integrin antibody, Arvin®, Atacand®, CerAxon®, Cerebyx®, Ceresine®, Cerestat®, Cervene®, Coumadin®, Fiblast®, Fraxiparine®, Freedox®, Innohep®, Kabikinase®, Klerval®, LeukArrest®, Lipitor®, Lovenox®, Neurogard®, Nimotop®, Orgaran®, Persantine®, Plavix®, Prolyse®, Prosynap®, ReoPro®, Selfotel®, Sibelium®, Streptase®, Streptokinase, Sygen®, Ticlid®, Trental®, Viprinex®, Warfarin, Zanaflex®, Zendra®), to preserve VF or VPI levels at or near non-Vascular Dementia values. The levels of one or more VFs or VPIs can be assayed.

In another embodiment, the methods and compositions of the present invention areused to screen candidates for a clinical study to identify individuals having vasculardementia; such individuals can then be excluded from the study or can be placed in aseparate cohort for treatment or analysis. If desired, the candidates can concurrently bescreened to identify individuals with Lewy Body disease and/or senile dementia;procedures for these screens are well known in the art (Harding and Halliday, *Neuropathol. Appl. Neurobiol.* (1998) 24:195-201).

5.5 Purification of VPIs

In particular aspects, the invention provides isolated mammalian VPIs, preferably human VPIs, and fragments thereof which comprise an antigenic determinant (*i.e.*, can be recognized by an antibody) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing. "Functionally active" as used herein refers to material displaying one or more functional activities associated with a full-length (wild-type) VPI, *e.g.*,

binding to a VPI substrate or VPI binding partner, antigenicity (binding to an anti-VPI antibody), immunogenicity, enzymatic activity and the like.

In specific embodiments, the invention provides fragments of a VPI comprising at least 5 amino acids, at least 10 amino acids, at least 50 amino acids, or at least 75 amino acids. Fragments lacking some or all of the regions of a VPI are also provided, as are proteins (*e.g.*, fusion proteins) comprising such fragments. Nucleic acids encoding the foregoing are provided.

Once a recombinant nucleic acid which encodes the VPI, a portion of the VPI, or a precursor of the VPI is identified, the gene product can be analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labeling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

The VPIs identified herein can be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

Alternatively, once a recombinant nucleic acid that encodes the VPI is identified, the entire amino acid sequence of the VPI can be deduced from the nucleotide sequence of the gene coding region contained in the recombinant nucleic acid. As a result, the protein can be synthesized by standard chemical methods known in the art (*e.g.*, see Hunkapiller et al, 1984, Nature 310:105-111).

In another alternative embodiment, native VPIs can be purified from natural sources, by standard methods such as those described above (*e.g.*, immunoaffinity purification).

In a preferred embodiment, VPIs are isolated by the Preferred Technology described supra. For preparative-scale runs, a narrow-range "zoom gel" having a pH range of 2 pH units or less is preferred for the isoelectric step, according to the method described in Westermeier, 1993, Electrophoresis in Practice (VCH, Weinheim, Germany), pp. 197-209 (which is incorporated herein by reference in its entirety); this modification permits a larger quantity of a target protein to be loaded onto the gel, and thereby increases the quantity of isolated VPI that can be recovered from the gel. When used in this way for preparative-scale runs, the Preferred Technology typically provides up to 100 ng, and can provide up to 1000 ng, of an isolated VPI in a single run. Those of skill in the art will appreciate that a zoom gel can be used in any separation strategy that employs gel isoelectric focusing.

The invention thus provides an isolated VPI, an isolated VPI-related polypeptide, and an isolated derivative or fragment of a VPI or a VPI-related polypeptide; any of the foregoing can be produced by recombinant DNA techniques or by chemical synthetic methods.

5.6 Isolation of DNA Encoding a VPI

Specific embodiments for the cloning of a gene encoding a VPI, are presented below by way of example and not of limitation.

The nucleotide sequences of the present invention, including DNA and RNA, and comprising a sequence encoding a VPI or a fragment thereof, or a VPI-related polypeptide, may be synthesized using methods known in the art, such as using conventional chemical approaches or polymerase chain reaction (PCR) amplification. The nucleotide sequences of the present invention also permit the identification and cloning of the gene encoding a VPI homolog or VPI ortholog including, for example, by screening cDNA libraries, genomic libraries or expression libraries.

For example, to clone a gene encoding a VPI by PCR techniques, anchored degenerate oligonucleotides (or a set of most likely oligonucleotides) can be designed for all VPI peptide fragments identified as part of the same protein. PCR reactions under a variety of conditions can be performed with relevant cDNA and genomic DNAs (*e.g.*, from brain tissue or from cells of the immune system) from one or more species. Also vectorette reactions can be performed on any available cDNA and genomic DNA using the oligonucleotides (which preferably are nested) as above. Vectorette PCR is a method that enables the amplification of specific DNA fragments in situations where the sequence of only one primer is known. Thus, it extends the application of PCR to stretches of DNA where the sequence information is only available at one end. (Arnold C, *PCR Methods Appl.* (1991) 1(1):39-42; Dyer K.D, *Biotechniques*, (1995) 19(4):550-2). Vectorette PCR may pe performed with probes that are, for example, anchored degenerate oligonucleotides (or most likely oligonucleotides) coding for VPI peptide fragments, using as a template a genomic library or cDNA library pools.

Anchored degenerate oligonucleotides (and most likely oligonucleotides) can be designed for all VPI peptide fragments. These oligonucleotides may be labelled and hybridized to filters containing cDNA and genomic DNA libraries. Oligonucleotides to different peptides from the same protein will often identify the same members of the library. The cDNA and genomic DNA libraries may be obtained from any suitable or desired mammalian species, for example from humans.

Nucleotide sequences comprising a nucleotide sequence encoding a VPI or VPI fragment of the present invention are useful for their ability to hybridize selectively with complementary stretches of genes encoding other proteins. Depending on the application, a variety of hybridization conditions may be employed to obtain nucleotide sequences at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identical, or 100% identical, to the sequence of a nucleotide encoding a VPI.

For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt or high temperature conditions. As used herein, "highly stringent conditions" means hybridization to filter-bound DNA in 0.5 M NaHPO4, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65 °C, and washing in 0.1xSSC/0.1% SDS at 68 °C (Ausubel F.M. et al, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3; incorporated herein by reference in its entirety.) For some applications, less stringent conditions for duplex formation are required. As used herein "moderately stringent conditions" means washing in 0.2xSSC/0.1% SDS at 42 °C (Ausubel et al, 1989, supra). Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be chosen depending on the desired results. In general, convenient hybridization temperatures in the presence of 50% formamide are: 42 °C for a probe which is 95 to 100% identical to the fragment of a gene encoding a VPI, 37 °C for 90 to 95% identity and 32 °C for 70 to 90% identity.

In the preparation of genomic libraries, DNA fragments are generated, some of which will encode parts or the whole of a VPI. Any suitable method for preparing DNA fragments may be used in the present invention. For example, the DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The DNA fragments can then be separated according to size by standard techniques, including but not limited to agarose and polyacrylamide gel electrophoresis, column chromatography and sucrose gradient centrifugation. The DNA fragments can then be inserted into suitable vectors, including but not limited to plasmids, cosmids, bacteriophages lambda or T4, and yeast artificial chromosome (YAC). (See, *e.g.*, Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II; Ausubel F.M. et al, eds., 1989, Current Protocols in

Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York). The genomic library may be screened by nucleic acid hybridization to labelled probe (Benton and Davis, *Science* (1977) 196:180; Grunstein and Hogness, *Proc. Natl. Acad. Sci. USA* (1975) 72:3961).

Based on the present description, the genomic libraries may be screened with labelled degenerate oligonucleotide probes corresponding to the amino acid sequence of any peptide of the VPI using optimal approaches well known in the art. Any probe used is at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides, at least 25 nucleotides, at least 30 nucleotides, at least 40 nucleotides, at least 50 nucleotides, at least 60 nucleotides, at least 70 nucleotides, at least 80 nucleotides, or at least 100 nucleotides. Preferably a probe is 10 nucleotides or longer, and more preferably 15 nucleotides or longer.

In Tables IV and V above, some VPIs disclosed herein were found to correspond to isoforms of previously identified proteins encoded by genes whose sequences are publicly known. (Sequence analysis and protein identification of VPIs was carried out using the methods described in Section 6.1.14). To screen such a gene, any probe may be used that is complementary to the gene or its complement; preferably the probe is 10 nucleotides or longer, more preferably 15 nucleotides or longer. The SWISS-PROT and trEMBL databases (held by the Swiss Institute of Bioinformatics (SIB) and the European Bioinformatics Institute (EBI) which are available at http://www.expasy.ch/) and the GenBank database (held by the National Institute of Health (NIH) which is available at http://www.ncbi.nlm.nih.gov/GenBank/) provide protein sequences for the VPIs listed in Tables IV and V under the following accession numbers and each sequence is incorporated herein by reference:

Table X. Nucleotide sequences encoding VPIs, VPI Related Proteins or ERPIs

VF#	VPI#	Accession Numbers of Identified Sequences
VF-4	VPI-2	P01034
VF-5	VPI-3	P41222
VF-12	VPI-6	P01024
VF-13	VPI-7	P10643
VF-14	VPI-8	P00747
VF-15	VPI-90	P10909
VF-16	VPI-9	P05090
VF-19	VPI-130	P01024
VF-20	VPI-91	P09571

VF#	VPI#	Accession Numbers of Identified Sequences
VF-20	VPI-92	P01024
VF-22	VPI-10	P01034
VF-25	VPI-131	P01034
VF-26	VPI-11	P01034
VF-27	VPI-93	P01034
VF-29	VPI-13	P41222
VF-32	VPI-94	P00747
VF-32	VPI-95	5453874 (gb)
VF-35	VPI-133	P02766
VF-36	VPI-14	P01034
VF-37	VPI-15	P01033
VF-38	VPI-96	P41222
VF-41	VPI-17	P01024
VF-41	VPI-97	P10909
VF-42	VPI-18	P41222
VF-43	VPI-19	P36955
VF-43	VPI-98	P02768
VF-44	VPI-21	Q12860
VF-45	VPI-22	P07602
VF-46	VPI-23	P01024
VF-47	VPI-24	P01008
VF-47	VPI-99	P02766
VF-48	VPI-25	P02766
VF-50	VPI-27	P01019
VF-50	VPI-28	P02774
VF-52	VPI-29	Q99435
VF-53	VPI-31	P01024
VF-54	VPI-100	P02662
VF-55	VPI-32	P36955
VF-55	VPI-33	5802984 (gb)
VF-57	VPI-37	P36222
VF-58	VPI-38	7662374 (gb)
VF-60	VPI-40	P98160
VF-64	VPI-43	P01019
VF-66	VPI-46	P41222
VF-68	VPI-48	P01011

VF#	VPI#	Accession Numbers of Identified Sequences
VF-68	VPI-49	P02768
VF-68	VPI-50	P02790
VF-92	VPI-56	P07360
VF-92	VPI-57	P01034
VF-93	VPI-146	88052
VF-94	VPI-147	P01028
VF-95	VPI-108	P04075
VF-97	VPI-58	P02023
VF-98	VPI-61	P01034
VF-98	VPI-109	P02023
VF-99	VPI-62	P19652
VF-100	VPI-63	P01024
VF-101	VPI-64	P02763
VF-102	VPI-65	P02768
VF-102	VPI-66	P02790
VF-104	VPI-272	P41222
VF-105	VPI-110	P05090
VF-106	VPI-111	7019363 (gb),
VF-107	VPI-273	Q92876
VF-108	VPI-148	P01922
VF-109	VPI-112	P01028
VF-112	VPI-149	P01028
VF-118	VPI-150	P41222
VF-122	VPI-67	P00918
VF-122	VPI-68	229528
VF-123	VPI-152	P41222
VF-127	VPI-69	P41222
VF-127	VPI-70	P01034
VF-132	VPI-113	P02649
VF-134	VPI-114	P23144
VF-135	VPI-269	P36222
VF-136	VPI-71	P41222
VF-137	VPI-270	P30086
VF-138	VPI-72	P41222
VF-139	VPI-73	P41222
VF-140	VPI-271	P36955

VF#	VPI#	Accession Numbers of Identified Sequences
VF-141	VPI-75	809027 (gb)
VF-142	VPI-77	P16035
VF-143	VPI-78	P41222
VF-144	VPI-79	P01024
VF-145	VPI-80	P07358
VF-147	VPI-115	M26880.1
VF-147	VPI-116	P55052
VF-148	VPI-117	P13645
VF-149	VPI-81	P16035
VF-150	VPI-118	P01028
VF-151	VPI-82	P41222
VF-152	VPI-153	P02768
VF-153	VPI-83	P01024
VF-153	VPI-119	P02749
VF-154	VPI-84	P01024
VF-155	VPI-120	P00736
VF-156	VPI-85	P01027
VF-157	VPI-121	P41222
VF-158	VPI-122	P41222
VF-159	VPI-123	P00734
VF-160	VPI-124	899271
VF-161	VPI-268	P06396
VF-163	VPI-87	P01344
VF-164	VPI-125	P41222
VF-166	VPI-154	P08253
VF-166	VPI-155	P02748
VF-166	VPI-156	9368806, 9368807,
VF-168	VPI-88	P02766
VF-170	VPI-127	P10909
VF-170	VPI-128	P05090
VF-170	VPI-129	P02790
VF-171	VPI-158	P01034
VF-172	VPI-159	P41222
VF-177	VPI-160	P51693
VF-180	VPI-161	P36222
VF-181	VPI-162	P01028

VF#	VPI#	Accession Numbers of Identified Sequences
VF-188	VPI-163	P06396
VF-189	VPI-164	P01028
VF-193	VPI-165	P01034
VF-195	VPI-166	P01028
VF-203	VPI-167	P05155
VF-204	VPI-168	P01028
VF-207	VPI-169	P01028
VF-209	VPI-170	P01024
VF-209	VPI-171	P05546
VF-210	VPI-172	P00734
VF-212	VPI-173	P01034
VF-213	VPI-174	181482
VF-213	VPI-175	P01019
VF-216	VPI-176	6651381
VF-216	VPI-177	P00450
VF-216	VPI-178	P01876
VF-219	VPI-179	P01034
VF-222	VPI-180	P10643
VF-223	VPI-181	2117873
VF-226	VPI-182	P02766
VF-227	VPI-183	P04264
VF-227	VPI-184	Q14118
VF-227	VPI-185	Q12805
VF-228	VPI-186	P23142
VF-230	VPI-187	2745741
VF-232	VPI-188	P01028
VF-234	VPI-189	P02790
VF-234	VPI-190	P02768
VF-235	VPI-191	7662374
VF-237	VPI-192	P23142
VF-239	VPI-193	P04004
VF-241	VPI-194	P01028
VF-242	VPI-195	P51693
VF-243	VPI-196	P05155
VF-245	VPI-197	P01023
VF-246	VPI-198	P23142

VF#	VPI#	Accession Numbers of Identified Sequences
VF-247	VPI-199	P04264
VF-249	VPI-200	410564
VF-250	VPI-201	2745741
VF-253	VPI-202	P16035
VF-254	VPI-203	P05090
VF-255	VPI-204	P00450
VF-256	VPI-205	P36955
VF-256	VPI-206	P04264
VF-258	VPI-207	P23142
VF-260	VPI-208	P30086
VF-261	VPI-209	P02763
VF-261	VPI-210	O14791
VF-262	VPI-211	P01034
VF-263	VPI-212	P02647
VF-264	VPI-213	P05090
VF-275	VPI-214	P02763
VF-277	VPI-215	P02763
VF-280	VPI-216	P01034
VF-281	VPI-217	P01034
VF-282	VPI-218	P02768
VF-287	VPI-219	P41222
VF-290	VPI-220	P01034
VF-291	VPI-221	P01034
VF-292	VPI-222	Q12805
VF-295	VPI-223	P05090
VF-299	VPI-224	P00450
VF-302	VPI-225	223918
VF-303	VPI-226	P01034
VF-305	VPI-227	P02765
VF-306	VPI-228	P02768
VF-309	VPI-229	P02766
VF-311	VPI-230	P02768
VF-313	VPI-231	P05090
VF-314	VPI-232	P01028
VF-316	VPI-233	P36955
VF-319	VPI-234	P06396

VF#	VPI#	Accession Numbers of Identified Sequences
VF-324	VPI-235	P15169
VF-324	VPI-236	401767
VF-329	VPI-237	P41222
VF-330	VPI-238	115647.1
VF-331	VPI-239	P10643
VF-332	VPI-240	P02768
VF-333	VPI-241	P01028
VF-333	VPI-242	P36955
VF-334	VPI-243	P01884
VF-335	VPI-244	P08735
VF-336	VPI-245	P41222
VF-337	VPI-246	339568
VF-340	VPI-247	P02790
VF-341	VPI-248	P35908
VF-342	VPI-249	P41222
VF-343	VPI-250	P36955
VF-344	VPI-251	P13645
VF-346	VPI-252	P41222
VF-346	VPI-253	P02768
VF-348	VPI-254	P06396
VF-349	VPI-255	P29622
VF-351	VPI-256	P41222
VF-352	VPI-257	P16035
VF-353	VPI-258	Q03591
VF-356	VPI-259	P02774
VF-356	VPI-260	P01019
VF-359	VPI-261	P04217
VF-360	VPI-262	P05090
VF-361	VPI-263	P41222
VF-362	VPI-264	P41222
VF-365	VPI-265	AK026519.1
VF-368	VPI-266	P02768
VF-370	VPI-267	P05090
ERF-1	ERPI-1	P01028
ERF-2	ERPI-2	P01019

For any VPI, degenerate probes, or probes taken from the sequences described above by accession number may be used for screening. In the case of degenerate probes, they can be constructed from the partial amino sequence information obtained from tandem mass spectra of tryptic digest peptides of the VPI. To screen such a gene, any probe may be used that is complementary to the gene or its complement; preferably the probe is 10 nucleotides or longer, more preferably 15 nucleotides or longer. When a library is screened, clones with insert DNA encoding the VPI or a fragment thereof will hybridize to one or more members of the corresponding set of degenerate oligonucleotide probes (or their complement). Hybridization of such oligonucleotide probes to genomic libraries is carried out using methods known in the art. For example, hybridization with one of the above-mentioned degenerate sets of oligonucleotide probes, or their complement (or with any member of such a set, or its complement) can be performed under highly stringent or moderately stringent conditions as defined above, or can be carried out in 2X SSC, 1.0% SDS at 50 °C and washed using the washing conditions described supra for highly stringent or moderately stringent hybridization.

In yet another aspect of the invention, clones containing nucleotide sequences encoding the entire VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide any of the foregoing may also be obtained by screening expression libraries. For example, DNA from the relevant source is isolated and random fragments are prepared and ligated into an expression vector (*e.g.*, a bacteriophage, plasmid, phagemid or cosmid) such that the inserted sequence in the vector is capable of being expressed by the host cell into which the vector is then introduced. Various screening assays can then be used to select for the expressed VPI or VPI-related polypeptides. In one embodiment, the various anti-VPI antibodies of the invention can be used to identify the desired clones using methods known in the art. See, for example, Harlow and Lane, 1988 Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Appendix IV. Colonies or plaques from the library are brought into contact with the antibodies to identify those clones that bind antibody.

In an embodiment, colonies or plaques containing DNA that encodes a VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide can be detected using DYNA Beads according to Olsvick et al, 29th ICAAC, Houston, Tex. 1989, incorporated herein by reference. Anti-VPI antibodies are crosslinked to tosylated DYNA Beads M280, and these antibody-containing beads are then contacted with colonies or plaques expressing recombinant polypeptides. Colonies or plaques expressing a VPI or VPI-related polypeptide are identified as any of those that bind the beads.

Alternatively, the anti-VPI antibodies can be nonspecifically immobilized to a suitable support, such as silica or Celite® resin. This material is then used to adsorb to bacterial colonies expressing the VPI protein or VPI-related polypeptide as described herein.

In another aspect, PCR amplification may be used to isolate from genomic DNA a substantially pure DNA (*i.e.*, a DNA substantially free of contaminating nucleic acids) encoding the entire VPI or a part thereof. Preferably such a DNA is at least 95% pure, more preferably at least 99% pure. Oligonucleotide sequences, degenerate or otherwise, that correspond to peptide sequences of VPIs disclosed herein can be used as primers.

PCR can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp® or AmpliTaq DNA polymerase). One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the degenerate primers and the corresponding sequences in the DNA. After successful amplification of a segment of the sequence encoding a VPI, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a complete genomic clone. This, in turn, will permit the determination of the gene's complete nucleotide sequence, the analysis of its expression, and the production of its protein product for functional analysis, as described *infra*.

The gene encoding a VPI can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified DNA encoding a VPI of another species (*e.g.*, mouse, human). Immunoprecipitation analysis or functional assays (*e.g.*, aggregation ability *in vitro*; binding to receptor) of the *in vitro* translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies that specifically recognize a VPI. A radiolabelled cDNA encoding a VPI can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabelled mRNA or cDNA may then be used as a probe to identify the DNA fragments encoding a VPI from among other genomic DNA fragments.

Alternatives to isolating genomic DNA encoding a VPI include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA that encodes the VPI. For example, RNA for cDNA cloning of the gene encoding a VPI can be isolated from cells that express the VPI. Those skilled in the art will understand

from the present description that other methods may be used and are within the scope of the invention.

Any suitable eukaryotic cell can serve as the nucleic acid source for the molecular cloning of the gene encoding a VPI. The nucleic acid sequences encoding the VPI can be isolated from vertebrate, mammalian, primate, human, porcine, bovine, feline, avian, equine, canine or murine sources. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (See, *e.g.*, Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences.

The identified and isolated gene or cDNA can then be inserted into any suitable cloning vector. A large number of vector-host systems known in the art may be used. As those skilled in the art will appreciate, the only limitation is that the vector system chosen be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, plasmids such as PBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene) or modified viruses such as adenoviruses, adeno-associated viruses or retroviruses. The insertion into a cloning vector can be accomplished, for example, by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and the gene encoding a VPI may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated gene encoding the VPI, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from

the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The nucleotide sequences of the present invention include nucleotide sequences encoding amino acid sequences with substantially the same amino acid sequences as native VPIs, nucleotide sequences encoding amino acid sequences with functionally equivalent amino acids, nucleotide sequences encoding VPIs, a fragments of VPIs, VPI- related polypeptides, or fragments of VPI-related polypeptides.

In a specific embodiment, an isolated nucleic acid molecule encoding a VPI- related polypeptide can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of a VPI such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Standard techniques known to those of skill in the art can be used to introduce mutations, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed and the activity of the protein can be determined.

5.7 Expression of DNA Encoding VPIs

The nucleotide sequence coding for a VPI, a VPI analog, a VPI-related peptide, or a fragment or other derivative of any of the foregoing, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and

translational signals can also be supplied by the native gene encoding the VPI or its flanking regions, or the native gene encoding the VPI- related polypeptide or its flanking regions. A variety of host-vector systems may be utilized in the present invention to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In specific embodiments, a nucleotide sequence encoding a human gene (or a nucleotide sequence encoding a functionally active portion of a human VPI) is expressed. In yet another embodiment, a fragment of a VPI comprising a domain of the VPI is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional and translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a VPI or fragment thereof may be regulated by a second nucleic acid sequence so that the VPI or fragment is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a VPI may be controlled by any promoter or enhancer element known in the art. Promoters which may be used to control the expression of the gene encoding a VPI or a VPI- related polypeptide include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon *Nature* (1981) 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al, *Cell* (1980) 22:787-797), the herpes thymidine kinase promoter (Wagner et al, *Proc. Natl. Acad. Sci. USA* (1981) 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al, *Nature* (1982) 296:39-42), the tetracycline (Tet) promoter (Gossen et al, *Proc. Nat. Acad. Sci. USA* (1995) 89:5547-5551); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al, *Proc. Natl. Acad. Sci. USA* (1978) 75:3727-3731), or the tac promoter (DeBoer, et al, *Proc. Natl. Acad. Sci. USA* (1983) 80:21-25; see also "Useful proteins from recombinant bacteria" in *Scientific American* (1980) 242:74-94); plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al, *Nature* (1984) 310(5973):115-20) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al, *Nucl. Acids Res.* (1981) 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-

Estrella et al, *Nature* (1984) 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al, *Cell* (1984) 38:639-646; Ornitz et al, *Cold Spring Harbor Symp. Quant. Biol.* (1986) 50:399-409; MacDonald, *Hepatology* (1987) 7:425- 515); insulin gene control region which is active in pancreatic beta cells (Hanahan, *Nature* (1985) 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al, *Cell* (1984) 38:647-658; Adames et al, 1985, Nature 318:533-538; Alexander et al, *Mol. Cell. Biol.* (1987) 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al, *Cell* (1986) 45:485-495), albumin gene control region which is active in liver (Pinkert et al, *Genes and Devel.* (1987) 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al, *Mol. Cell. Biol.* (1985) 5:1639-1648; Hammer et al, *Science* (1987) 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, *Genes and Devel.* (1987) 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al, *Nature* (1985) 315:338-340; Kollias et al, *Cell* (1986) 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al, *Cell* (1987) 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, *Nature* (1985) 314:283-286); neuronal-specific enolase (NSE) which is active in neuronal cells (Morelli et al, *Gen. Virol.* (1999) 80:571-83); brain-derived neurotrophic factor (BDNF) gene control region which is active in neuronal cells (Tabuchi et al, *Biochem. Biophysic. Res. Com.* (1998) 253:818-823); glial fibrillary acidic protein (GFAP) promoter which is active in astrocytes (Gomes et al, *Braz J Med Biol Res* (1999) 32(5):619-631; Morelli et al, *Gen. Virol.* (1999) 80:571-83) and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al, *Science* (1986) 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to a VPI-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning a VPI or a VPI-related polypeptide coding sequence into the EcoRI restriction site of each of the three pGEX vectors (Glutathione S-Transferase expression vectors; Smith and Johnson, *Gene*

(1988) 7:31-40). This allows for the expression of the VPI product or VPI-related polypeptide from the subclone in the correct reading frame.

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the VPI coding sequence or VPI-related polypeptide coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts. (*e.g.*, see Logan & Shenk, *Proc. Natl. Acad. Sci. USA* (1984) 81:355-359). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al, *Methods in Enzymol.* (1987) 153:51-544).

Expression vectors containing inserts of a gene encoding a VPI or a VPI- related polypeptide can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a gene encoding a VPI inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted gene encoding a VPI. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a gene encoding a VPI in the vector. For example, if the gene encoding the VPI is inserted within the marker gene sequence of the vector, recombinants containing the gene encoding the VPI insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the gene product (*i.e.*, VPI) expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the VPI in *in vitro* assay systems, *e.g.*, binding with anti-VPI antibody.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered VPI or VPI-related polypeptide may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system will produce an unglycosylated product and expression in yeast will produce a glycosylated product. Eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, Hela, COS, MDCK, 293, 3T3, WI38, and in particular, neuronal cell lines such as, for example, SK-N-AS, SK-N-FI, SK-N-DZ human neuroblastomas (Sugimoto et al, *J. Natl. Cancer Inst.* (1984) 73: 51-57), SK-N-SH human neuroblastoma (Biochim. Biophys. Acta, 1982, 704: 450-460), Daoy human cerebellar medulloblastoma (He et al, *Cancer Res.* (1992) 52: 1144-1148) DBTRG-05MG glioblastoma cells (Kruse et al, *In vitro Cell. Dev. Biol.* (1992) 28A: 609-614), IMR-32 human neuroblastoma (*Cancer Res.*, (1970) 30:2110-2118), 1321N1 human astrocytoma (*Proc. Natl Acad. Sci. USA* (1977) 74:4816), MOG-G-CCM human astrocytoma (*Br. J. Cancer*, (1984) 49:269), U87MG human glioblastoma-astrocytoma (*Acta Pathol. Microbiol. Scand.*, (1968) 74: 465-486), A172 human glioblastoma (Olopade et al, *Cancer Res.* (1992) 52:2523- 2529), C6 rat glioma cells (Benda et al, *Science* (1968) 161:370-371), Neuro-2a mouse neuroblastoma (*Proc. Natl. Acad. Sci. USA*, (1970) 65: 129-136), NB41A3 mouse neuroblastoma (*Proc. Natl. Acad. Sci. USA*, (1962) 48:1184-1190), SCP sheep choroid plexus (Bolin et al, *J. Virol. Methods* (1994) 48: 211-221), G355-5, PG-4 Cat normal astrocyte (Haapala et al, *J. Virol.* (1985) 53:827-833), Mpf ferret brain (Trowbridge et al, *In vitro* (1982) 18:952-960), and normal cell lines such as, for example, CTX TNA2 rat normal cortex brain (Radany et al, *Proc. Natl. Acad. Sci. USA* (1992) 89:6467-6471) such as, for example, CRL7030 and Hs578Bst. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the differentially expressed or pathway gene protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate

expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched medium, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the differentially expressed or pathway gene protein. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the differentially expressed or pathway gene protein.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al, *Cell* (1977) 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, *Proc. Natl. Acad. Sci. USA* (1962) 48:2026), and adenine phosphoribosyltransferase (Lowy, et al, *Cell* (1980) 22:817) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al, *Proc. Natl. Acad. Sci. USA* (1980) 77:3567; O'Hare, et al, *Proc. Natl. Acad. Sci. USA* (1981) 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, *Proc. Natl. Acad. Sci. USA* (1981) 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre- Garapin, et al, *J. Mol. Biol.* (1981) 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al, *Gene* (1984) 30:147) genes.

In other specific embodiments, the VPI, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence). For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, or any combination thereof and portions thereof) resulting in chimeric polypeptides. Such fusion proteins may facilitate purification, increase half-life *in vivo*, and enhance the delivery of an antigen across an epithelial barrier to the immune system. An increase in the half-life *in vivo* and facilitated purification has been shown for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. See, *e.g.*, EP 394,827; Traunecker et al, *Nature*, (1988) 331:84-86 . Enhanced delivery of an antigen across the epithelial barrier to the immune system has been demonstrated for antigens (*e.g.*, insulin) conjugated to an FcRn

binding partner such as IgG or Fc fragments (see, *e.g.*, PCT publications WO 96/22024 and WO 99/04813).

Nucleic acids encoding a VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide can be fused to an epitope tag (*e.g.*, the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht et al, allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al, *Proc. Natl. Acad. Sci. USA* (1991) 88:8972-897).

Fusion proteins can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, a fusion protein may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer.

Both cDNA and genomic sequences can be cloned and expressed.

5.8 Domain Structure of VPIs

Domains of some VPIs are known in the art and have been described in the scientific literature. Moreover, domains of a VPI can be identified using techniques known to those of skill in the art. For example, one or more domains of a VPI can be identified by using one or more of the following programs: ProDom, TMpred, and SAPS. ProDom compares the amino acid sequence of a polypeptide to a database of compiled domains (see, *e.g.*, http://www.toulouse.inra.fr/prodom.html; Corpet F., Gouzy J. & Kahn D., *Nucleic Acids Res.*, (1999) 27:263-267). TMpred predicts membrane-spanning regions of a polypeptide and their orientation. This program uses an algorithm that is based on the statistical analysis of TMbase, a database of naturally occuring transmembrane proteins (see, *e.g.*, http://www.ch.embnet.org/software/TMPRED_form.html; Hofmann & Stoffel. (1993) "TMbase - A database of membrane spanning proteins segments." Biol. Chem. Hoppe-Seyler 347,166). The SAPS program analyzes polypeptides for statistically significant features like charge-clusters, repeats, hydrophobic regions, compositional domains (see, *e.g.*, Brendel et al, *Proc. Natl. Acad. Sci. USA* (1992) 89: 2002-2006). Thus, based on the present description, the skilled artisan can identify domains of a VPI having enzymatic or binding activity, and further can identify nucleotide sequences encoding such domains. These nucleotide sequences can then be used for recombinant expression of a VPI fragment that retains the enzymatic or binding activity of the VPI.

Based on the present description, the skilled artisan can identify domains of a VPI having enzymatic or binding activity, and further can identify nucleotide sequences encoding such domains. These nucleotide sequences can then be used for recombinant expression of VPI fragments that retain the enzymatic or binding activity of the VPI.

In one embodiment, a VPI has an amino acid sequence sufficiently similar to an identified domain of a known polypeptide. As used herein, the term "sufficiently similar" refers to a first amino acid or nucleotide sequence which contains a sufficient number of identical or equivalent (*e.g.*, with a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have or encode a common structural domain or common functional activity or both.

A VPI domain can be assessed for its function using techniques well known to those of skill in the art. For example, a domain can be assessed for its kinase activity or for its ability to bind to DNA using techniques known to the skilled artisan. Kinase activity can be assessed, for example, by measuring the ability of a polypeptide to phosphorylate a substrate. DNA binding activity can be assessed, for example, by measuring the ability of a polypeptide to bind to a DNA binding element in a electromobility shift assay. In a preferred embodiment, the function of a domain of a VPI is determined using an assay described in one or more of the references identified in Table XI, *infra*.

5.9 Production of Antibodies to VPIs

According to the invention a VPI, VPI analog, VPI-related protein or a fragment or derivative of any of the foregoing may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such immunogens can be isolated by any convenient means, including the methods described above. Antibodies of the invention include, but are not limited to polyclonal, monoclonal, bispecific, humanized or chimeric antibodies, single chain antibodies, Fab fragments and F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds an antigen. The immunoglobulin molecules of the invention can be of any class (*e.g.*, IgG, IgE, IgM, IgD and IgA) or subclass of immunoglobulin molecule.

In one embodiment, antibodies that recognize gene products of genes encoding VPIs are publicly available. For example, antibodies that recognize these VPIs and/or their isoforms include antibodies recognizing: VPI-2, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-14, VPI-15, VPI-17, VPI-23, VPI-24, VPI-25, VPI-27, VPI-31, VPI-43, VPI-48, VPI-49, VPI-50, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-70, VPI-77, VPI-79, VPI-80, VPI-81, VPI-83, VPI-84, VPI-87, VPI-88, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-97, VPI-98, VPI-99, VPI-109, VPI-110, VPI-112, VPI-113, VPI-117, VPI-118, VPI-120, VPI-123, VPI-127, VPI-128, VPI-129, VPI-130, VPI-131, VPI-133, VPI-147, VPI-149, VPI-153, VPI-158, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-175, VPI-178, VPI-179, VPI-180, VPI-182, VPI-183, VPI-188, VPI-189, VPI-190, VPI-194, VPI-199, VPI-202, VPI-203, VPI-206, VPI-209, VPI-211, VPI-212, VPI-213, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-220, VPI-221, VPI-223, VPI-226, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-234, VPI-239, VPI-240, VPI-241, VPI-247, VPI-251, VPI-253, VPI-254, VPI-255, VPI-257, VPI-258, VPI-260, VPI-261, VPI-262, VPI-266, VPI-267, VPI-268, which antibodies can be purchased from commercial sources as shown in Table VII above. In another embodiment, methods known to those skilled in the art are used to produce antibodies that recognize a VPI, a VPI analog, a VPI-related polypeptide, or a derivative or fragment of any of the foregoing.

In one embodiment of the invention, antibodies to a specific domain of a VPI are produced. In a specific embodiment, hydrophilic fragments of a VPI are used as immunogens for antibody production.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of a VPI, one may assay generated hybridomas for a product which binds to a VPI fragment containing such domain. For selection of an antibody that specifically binds a first VPI homolog but which does not specifically bind to (or binds less avidly to) a second VPI homolog, one can select on the basis of positive binding to the first VPI homolog and a lack of binding to (or reduced binding to) the second VPI homolog. Similarly, for selection of an antibody that specifically binds a VPI but which does not specifically bind to (or binds less avidly to) a different isoform of the same protein (such as a different glycoform having the same core peptide as the VPI), one can select on the basis of positive binding to the VPI and a lack of binding to (or reduced binding to) the different isoform (*e.g.*, a different glycoform). Thus, the present invention

provides an antibody (preferably a monoclonal antibody) that binds with greater affinity (preferably at least 2-fold, more preferably at least 5-fold still more preferably at least 10-fold greater affinity) to a VPI than to a different isoform or isoforms (*e.g.*, glycoforms) of the VPI.

Polyclonal antibodies that may be used in the methods of the invention are heterogeneous populations of antibody molecules derived from the sera of immunized animals. Unfractionated immune serum can also be used. Various procedures known in the art may be used for the production of polyclonal antibodies to a VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide. In a particular embodiment, rabbit polyclonal antibodies to an epitope of a VPI or a VPI- related polypeptide can be obtained. For example, for the production of polyclonal or monoclonal antibodies, various host animals can be immunized by injection with the native or a synthetic (*e.g.*, recombinant) version of a VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide, including but not limited to rabbits, mice, rats, etc. The Preferred Technology described herein provides isolated VPIs suitable for such immunization. If the VPI is purified by gel electrophoresis, the VPI can be used for immunization with or without prior extraction from the polyacrylamide gel. Various adjuvants may be used to enhance the immunological response, depending on the host species, including, but not limited to, complete or incomplete Freund's adjuvant, a mineral gel such as aluminum hydroxide, surface active substance such as lysolecithin, pluronic polyol, a polyanion, a peptide, an oil emulsion, keyhole limpet hemocyanin, dinitrophenol, and an adjuvant such as BCG (bacille Calmette-Guerin) or corynebacterium parvum. Additional adjuvants are also well known in the art.

For preparation of monoclonal antibodies (mAbs) directed toward a VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (*Nature* (1975) 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al, *Immunology Today* (1983) 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al, (1985) in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAbs of the invention may be cultivated *in vitro* or *in vivo*. In an

additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing known technology (PCT/US90/02545, incorporated herein by reference).

The monoclonal antibodies include but are not limited to human monoclonal antibodies and chimeric monoclonal antibodies (*e.g.*, human-mouse chimeras). A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a human immunoglobulin constant region and a variable region derived from a murine mAb. (See, *e.g.*, Cabilly et al, U.S. Patent No. 4,816,567; and Boss et al, U.S. Patent No. 4,816397, which are incorporated herein by reference in their entirety.) Humanized antibodies are antibody molecules from non- human species having one or more complementarily determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. (See, *e.g.*, Queen, U.S. Patent No. 5,585,089, which is incorporated herein by reference in its entirety.)

Chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better et al, 1988, Science 240:1041-1043; Liu et al, *Proc. Natl. Acad. Sci. USA* (1987) 84:3439-3443; Liu et al, *J. Immunol.* (1987) 139:3521-3526; Sun et al, *Proc. Natl. Acad. Sci. USA* (1987) 84:214-218; Nishimura et al, *Canc. Res.* (1987) 47:999-1005; Wood et al, *Nature* (1985) 314:446-449; and Shaw et al, *J. Natl. Cancer Inst.* (1988) 80:1553-1559; Morrison, *Science* (1985) 229:1202-1207; Oi et al, *Bio/Techniques* (1986) 4:214; U.S. Patent 5,225,539; Jones et al, *Nature* (1986) 321:552-525; Verhoeyan et al, *Science* (1988) 239:1534; and Beidler et al, *J. Immunol.* (1988) 141:4053-4060.

Completely human antibodies are particularly desirable for therapeutic treatment of human subjects. Such antibodies can be produced using transgenic mice which are incapable of expressing endogenous immunoglobulin heavy and light chains genes, but which can express human heavy and light chain genes. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of a VPI of the invention. Monoclonal antibodies directed against the antigen can be obtained using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human

antibodies, see Lonberg and Huszar (*Int. Rev. Immunol.* (1995) 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, *e.g.*, U.S. Patent 5,625,126; U.S. Patent 5,633,425; U.S. Patent 5,569,825; U.S. Patent 5,661,016; and U.S. Patent 5,545,806. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al, *Bio/technology* (1994) 12:899-903).

The antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles that carry the polynucleotide sequences encoding them. In a particular, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, *e.g.*, using labelled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al, *J. Immunol. Methods* (1995) 182:41-50 ; Ames et al, *J. Immunol. Methods* (1995) 184:177-186 ; Kettleborough et al, *Eur. J. Immunol.* (1994) 24:952-958 ; Persic et al, *Gene* (1997) 187 9-18 ; Burton et al, *Advances in Immunology* (1994) 57:191-280 ; PCT Application No. PCT/GB91/01134; PCT Publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired

host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, *e.g.*, as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax et al, BioTechniques 12(6):864-869 (1992); and Sawai et al, (1995) AJRI 34:26-34 ; and Better et al, *Science* (1988) 240:1041-1043 (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston et al, Methods in Enzymology 203:46-88 (1991); Shu et al, *Proc. Natl. Sci Acad. USA* (1993) 90:7995-7999; and Skerra et al, *Science* (1988) 240:1038-1040 .

The invention further provides for the use of bispecific antibodies, which can be made by methods known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Milstein et al, *Nature* (1983) 305:537-539). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al, *EMBO J.* (1991) 10:3655-3659 .

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published March 3,1994. For further details for generating bispecific antibodies see, for example, Suresh et al, *Methods in Enzymology* (1986) 121:210.

The invention provides functionally active fragments, derivatives or analogs of the anti-VPI immunoglobulin molecules. Functionally active means that the fragment, derivative or analog is able to elicit anti-anti-idiotype antibodies (*i.e.*, tertiary antibodies) that recognize the same antigen that is recognized by the antibody from which the fragment, derivative or analog is derived. Specifically, in a preferred embodiment the antigenicity of the idiotype of the immunoglobulin molecule may be enhanced by deletion of framework and CDR sequences that are C-terminal to the CDR sequence that specifically recognizes the antigen. To determine which CDR sequences bind the antigen, synthetic peptides containing the CDR sequences can be used in binding assays with the antigen by any binding assay method known in the art.

The present invention provides antibody fragments such as, but not limited to, F(ab')2 fragments and Fab fragments. Antibody fragments which recognize specific epitopes may be generated by known techniques. F(ab')2 fragments consist of the variable region, the light chain constant region and the CH1 domain of the heavy chain and are generated by pepsin digestion of the antibody molecule. Fab fragments are generated by reducing the disulfide bridges of the F(ab')2 fragments. The invention also provides heavy chain and light chain dimers of the antibodies of the invention, or any minimal fragment thereof such as Fvs or single chain antibodies (SCAs) (*e.g.*, as described in U.S. Patent 4,946,778; Bird, *Science* (1988) 242:423-42; Huston et al, *Proc. Natl. Acad. Sci. USA* (1988) 85:5879-5883; and Ward et al, *Nature* (1989) 334:544-54), or any other molecule with the same specificity as the antibody of the invention. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in E. coli may be used (Skerra et al, *Science* (1988) 242:1038-1041).

In other embodiments, the invention provides fusion proteins of the immunoglobulins of the invention (or functionally active fragments thereof), for example in which the immunoglobulin is fused via a covalent bond (*e.g.*, a peptide bond), at either the N-terminus or the C-terminus to an amino acid sequence of another protein (or portion thereof, preferably at least 10, 20 or 50 amino acid portion of the protein) that is not the immunoglobulin. Preferably the immunoglobulin, or fragment thereof, is covalently linked to the other protein at the N-terminus of the constant domain. As stated above, such fusion proteins may facilitate purification, increase half-life *in vivo*, and enhance the delivery of an antigen across an epithelial barrier to the immune system.

The immunoglobulins of the invention include analogs and derivatives that are either modified, i.e, by the covalent attachment of any type of molecule as long as such covalent attachment that does not impair immunospecific binding. For example, but not by way of limitation, the derivatives and analogs of the immunoglobulins include those that have been further modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, etc. Additionally, the analog or derivative may contain one or more non-classical amino acids.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the VPIs of the invention, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

5.10 Expression Of Antibodies

The antibodies of the invention can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or by recombinant expression, and are preferably produced by recombinant expression techniques.

Recombinant expression of antibodies, or fragments, derivatives or analogs thereof, requires construction of a nucleic acid that encodes the antibody. If the nucleotide sequence of the antibody is known, a nucleic acid encoding the antibody may be assembled from chemically synthesized oligonucleotides (*e.g.*, as described in Kutmeier et al, *BioTechniques* (1994) 17:242), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding antibody, annealing and ligation of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, the nucleic acid encoding the antibody may be obtained by cloning the antibody. If a clone containing the nucleic acid encoding the particular antibody is not available, but the sequence of the antibody molecule is known, a nucleic acid encoding the antibody may be obtained from a suitable source (*e.g.*, an antibody cDNA library, or cDNA library generated from any tissue or cells expressing the antibody) by PCR amplification using synthetic primers hybridizable to the 3′ and 5′ ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence.

If an antibody molecule that specifically recognizes a particular antigen is not available (or a source for a cDNA library for cloning a nucleic acid encoding such an antibody), antibodies specific for a particular antigen may be generated by any method known in the art, for example, by immunizing an animal, such as a rabbit, to generate polyclonal antibodies or, more preferably, by generating monoclonal antibodies. Alternatively, a clone encoding at least the Fab portion of the antibody may be obtained by screening Fab expression libraries (*e.g.*, as described in Huse et al, *Science* (1989) 246:1275-1281) for clones of Fab fragments that bind the specific antigen or by screening antibody libraries (See, *e.g.*, Clackson et al, *Nature* (1991) 352:624; Hane et al, *Proc. Natl. Acad. Sci. USA* (1997) 94:4937).

Once a nucleic acid encoding at least the variable domain of the antibody molecule is obtained, it may be introduced into a vector containing the nucleotide sequence encoding the constant region of the antibody molecule (see, *e.g.*, PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464). Vectors containing the complete light or heavy chain for co-expression with the nucleic acid to allow the expression of a complete antibody molecule are also available. Then, the nucleic acid encoding the antibody can be used to introduce the nucleotide substitution(s) or deletion(s) necessary to substitute (or delete) the one or more variable region cysteine residues participating in an intrachain disulfide bond with an amino acid residue that does not contain a sulfhydyl group. Such modifications can be carried out by any method known in the art for the introduction of specific mutations or deletions in a nucleotide sequence, for example, but not limited to, chemical mutagenesis, *in vitro* site directed mutagenesis (Hutchinson et al, *J. Biol. Chem.* (1978) 253:6551), PCT based methods, etc.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al, *Proc. Natl. Acad. Sci.* (1984) 81:851-855; Neuberger et al, *Nature* (1984) 312:604-608; Takeda et al, *Nature* (1985) 314:452-454) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described supra, a

chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human antibody constant region, *e.g.*, humanized antibodies.

Once a nucleic acid encoding an antibody molecule of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the protein of the invention by expressing nucleic acid containing the antibody molecule sequences are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing an antibody molecule coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook et al, (1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and Ausubel et al, (eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY).

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody of the invention.

The host cells used to express a recombinant antibody of the invention may be either bacterial cells such as Escherichia coli, or, preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecule. In particular, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking et al, *Gene* (1986) 45:101; Cockett et al, *Bio/Technology* (1990) 8:2).

A variety of host-expression vector systems may be utilized to express an antibody molecule of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the antibody molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (*e.g.*, E. coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (*e.g.*, Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell

systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing antibody coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions comprising an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al, *EMBO J.* (1983) 2:1791), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, *Nucleic Acids Res.* (1985) 13:3101-3109; Van Heeke & Schuster, *J. Biol. Chem.* (1989) 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The antibody coding sequence may be cloned individually into non- essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). In mammalian host cells, a number of viral-based expression systems (*e.g.*, an adenovirus expression system) may be utilized.

As discussed above, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein.